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THE PHYSIOLOGY OF GROWTH IN APPLE FRUITS

VI. THE CONTROL OF RESPIRATION RATE AND SYNTHESIS

By Judith A. Pearson* and R. N. Robertson*

[*Manuscript received September 23, 1953*]

Summary

Experiments are described which test the hypothesis that the rate of respiration is controlled by the phosphate carrier system in apple tissue. The effects of 2,4-dinitrophenol and adenosine triphosphate on the respiration of cut tissue are consistent with the hypothesis that a more rapid utilization of energy-rich phosphate at a critical stage could result in the climacteric rise. The interrelations of starch, organic acid and nitrogen metabolism, and respiration are discussed. An increase in the activity of extracted respiratory enzymes at the time of the climacteric rise has been demonstrated.

I. Introduction

In earlier papers in this series (Robertson and Turner 1951; Pearson and Robertson 1952, 1953), Maskell's hypothesis was used to explain the rise in respiration rate, the climacteric rise, which occurs in Granny Smith apples developing on the tree. Maskell (1948) has pointed out that the removal of a controlling effect due to a low concentration of phosphate acceptors would result in an increase in respiration rate. Following our work on increase in protein content during the enlargement of the cells of the fruit, we suggested that there would be an increasing rate of turnover of phosphate carriers to meet the greater demand for energy for the protein maintenance. The more rapid turnover of phosphate carriers makes a higher concentration of acceptors available to take the energy-rich phosphates from the respiration process, which consequently increases in rate.

If the rate of respiration in pre-climacteric fruit were controlled by the concentration of phosphate acceptors, it would be expected that either an increase in phosphate acceptors supplied or an uncoupling of the oxidation system from the linked phosphate carriers would increase the rate. In climacteric fruit where the respiration had already risen, a marked increase in rate would not be expected. 2,4-Dinitrophenol is known to uncouple oxidation and phosphorylation and to stimulate oxygen uptake in tissues where the respiration rate is limited by the phosphate acceptor concentration.

Early in 1952, experiments were begun to test this hypothesis of the control of respiration. Recently Millerd, Bonner, and Biale (1953) have reported work along similar lines with the avocado in which the respiration rate is governed

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by a phosphorylative coupling mechanism. The respiratory rate of slices of pre-climacteric fruit but not that of climacteric fruit was increased by 2,4-dinitrophenol.

The purpose of this paper is to report results with apple tissue and with isolated respiratory particles, which support the hypothesis outlined above.

II. METHODS

(*a*) *Material and Sampling.*—Experiments were carried out on Granny Smith apples from trees at Bathurst during the two seasons 1951-52 and 1952-53. The sampling data are given in Table 1. The fruits were sent to the laboratory within 4 hr of picking. Except when the fruits were very small each fruit was weighed and six were put into glass shells for measurement of respiration rate. The remaining fruits were held at low temperature for 48 hr.

(*b*) *Respiration.*—The respiration rate of the whole fruit was measured at 25°C by the Pettenkofer method during the 70 hr after picking.

(*c*) *Preparation of Cut Tissue.*—After measurement on the whole fruits, four of the six were used in the Warburg apparatus for experiments on the respiration of freshly cut tissue. Cylindrical blocks of tissue were removed with a cork borer and disks 1 mm thick were cut from the cortex. One gram was used in each vessel. After weighing 1 g to the nearest whole disk, the disks were placed in a small piece of muslin and rinsed quickly in an isotonic solution of glucose (0.45M) and potassium dihydrogen phosphate (0.01M). They were spread, with as little handling as possible, on filter paper to dry the excess solution and then placed in the Warburg vessel in the isotonic solution (see Hackney 1946).

(*d*) *Measurements of the Effects of Adding 2,4-Dinitrophenol and Adenosine Triphosphate.*—The solutions in contact with the cut tissue were glucose (0.45M) and potassium dihydrogen phosphate (0.01M). 2,4-Dinitrophenol (DNP) and adenosine triphosphate (ATP) were added from the side-arm to give the desired concentration in contact with the tissue.

(*e*) *Preparation of Isolated Particles.*—The apples which had been kept at low temperature for about 48 hr after picking, were peeled and the flesh diced. The diced flesh was placed in a Waring Blendor with an equal volume of water or 0.45M sucrose solution and sufficient 5 per cent. potassium hydroxide was added during blending to maintain a neutral reaction with brom-thymol blue. Apple tissue has a pH of about 3 and it was found necessary to neutralize with potassium hydroxide during blending to obtain good activity. Water only was used as the blending medium in 1952. In the second season both water and 0.45M sucrose were used.

The tissue was blended for 30 sec and the homogenate squeezed through three layers of muslin. The muslin-squeezed homogenate was placed in 250 ml centrifuge tubes and spun at 1150g for 20 min. With the exception of the first four samples of the 1952-53 season, each tube contained the muslin-squeezed homogenate from 100 g fresh tissue. To obtain a washed residue, the residue in one tube was resuspended in 50 ml water and centrifuged at 1150g for another 20 min.

Table 1

SAMPLING DATA

Days from Full Blossom	1951-52 Full Blossom 7.x.1951		1952-53 Full Blossom 5.x.1952	
	Number of Fruits	Number of Trees	Number of Fruits	Number of Trees
16			55	1
29			76	1
43			20	1
64			10	1
92			12	12
99			12	12
106			12	12
114			12	12
120			12	12
127			12	12
134	10	1	12	12
141	10	1	12	12
148	10	1	12	12
155	10	1	12	12
162	10	1	12	12
169	10	1	12	12
176	10	1	—	—
177	—	—	12	12
183	10	1	—	—
184	—	—	12	12
191	10	1	12	12
197	10	1	—	—
199	—	—	12	12
204	10	1	—	—
205	—	—	12	4
211	10	1	—	—
212	—	—	12	4
218	10	1	—	—
219	—	—	12	4
225	10	1	—	—
226*	—	—	12	4
232	10	1	—	—
239	10	1	—	—
253	10	1	—	—
260	10	1	—	—
267	10	1	—	—

* Unfortunately no samples were obtained after this during 1952-53 as all the fruits were stolen from the trees.

(*f*) *Measurement of Particle Activity.*—Each residue in 1952 was resuspended in 3 ml water. In 1952-53 the residue was taken up in 3-7 ml of solution according to the total amount required in each experiment. In most experiments 5 ml were added and all the others were recalculated to correspond to the same dilution. In all experiments 1 ml of suspension was tested for oxygen uptake in a Warburg apparatus with sodium succinate, sodium citrate, or sodium malate as substrate at 27°C (1952) or 25°C (1953).

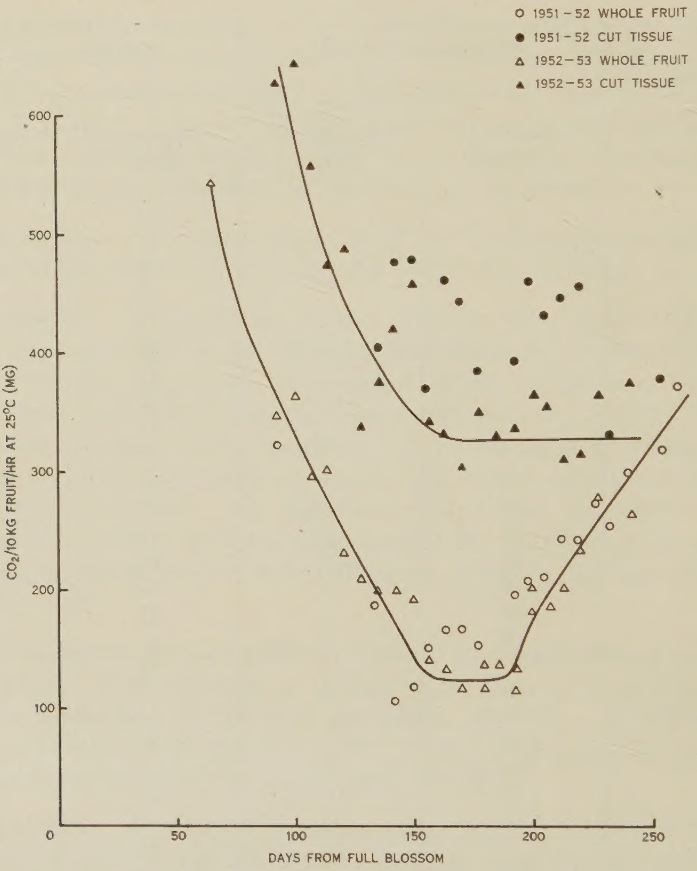


Fig. 1.—Changes in respiration rate (CO_2 output) per unit weight with time, comparing whole-fruit rates with cut-tissue rates in both seasons.

III. Results

(*a*) *Respiration Rates of Whole Fruits*

The results of the respiration rate measurements for whole fruits are given in Figure 1. Respiration per unit weight fell from a very high rate in the young fruit to a low rate at about 150 days from blossom. At 190 days it rose sharply and continued to rise till the end of the season. The same type of curve has been obtained in all other seasons.

(*b*) *Cut-tissue Experiments*

(i) *Cut-tissue Respiration.*—The cut-tissue respiration was measured as μl oxygen uptake per gram per hour at 25°C. On each sample the mean of three readings from each of four fruits was taken, with the exception of the first sample and of the last two samples of the 1951-52 season, when only two fruits were used.

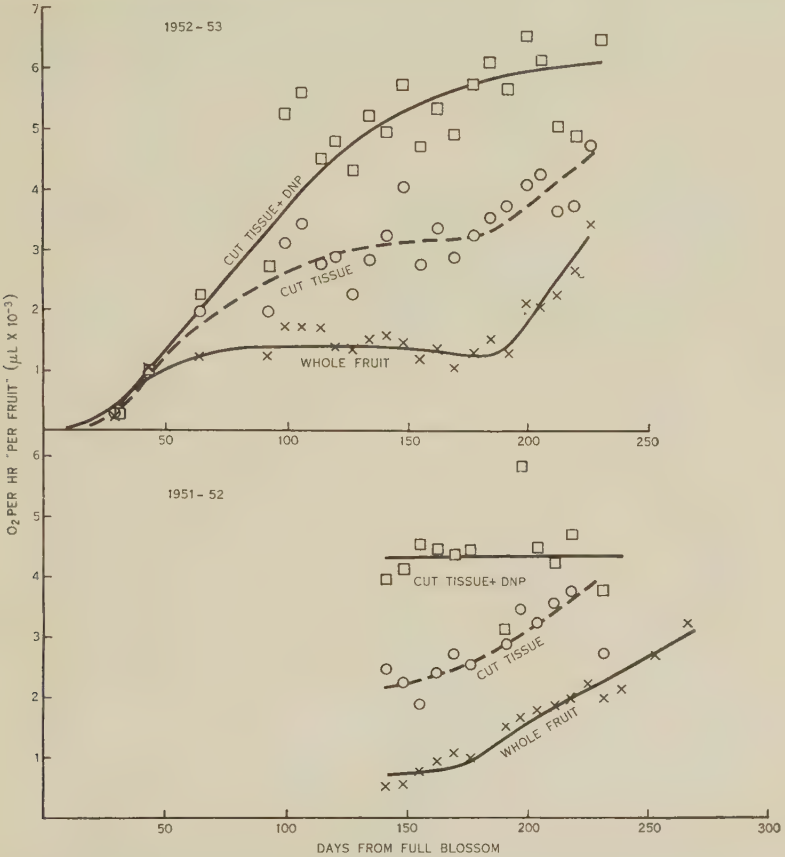


Fig. 2.—Comparison of the oxygen uptake of whole fruit (assuming R.Q. = 1) with the oxygen uptake of cut tissue with and without 2,4-dinitrophenol (DNP). The cut-tissue respiration has been corrected by multiplying by the weight to express it on a "per fruit" basis. The form of the curves would be that expected on a per cell basis since cell number does not change.

In Figure 1, the cut-tissue respiration is shown calculated as mg CO_2 per 10 kg per hour, assuming that R.Q. = 1. The cut-tissue respiration was very high in the young fruits but fell rapidly till 130 days, where it remained with no definite trend till the end of the season. The rate in 1951-52 was higher than in 1952-53.

The cut-tissue respiration may be compared with the whole-fruit rates when allowance is made for the changing weight during the growth of the fruit.

Earlier studies have shown that cell division is complete within 30 days of blossom. The change in respiration rate per fruit should therefore follow the same course as the change in respiration rate per cell. Respiration per fruit (μl O_2 uptake per fruit per hour, calculated assuming R.Q. = 1) is shown in Figure 2. On a comparable basis, the respiration per g of cut tissue must be multiplied by fruit weight and this curve is also shown in Figure 2. In the early samples the respiration per cell of cut tissue and of whole fruit are similar but by 100 days the cut-tissue respiration per cell is about double that per cell of the intact fruit. After about 190 days when the whole-fruit respiration showed a climacteric rise, the cut-tissue respiration also increased in both seasons.

(ii) *Action of 2,4-Dinitrophenol.*—In a preliminary experiment, 2,4-dinitrophenol was added from the side-arms in the following concentrations: 10, 5, 3, 1, 0.5, and 0.1 mg/100 ml. The respiration was inhibited by concentrations of 10 and 5 mg/100 ml, while 0.1 mg/100 ml gave no effect. Some stimulation was caused by 3 and 1 mg/100 ml, but the greatest stimulation in every trial was given by 0.5 mg/100 ml. This concentration, which corresponds to 0.5 mg/l in contact with the disks, was used throughout the experiments.

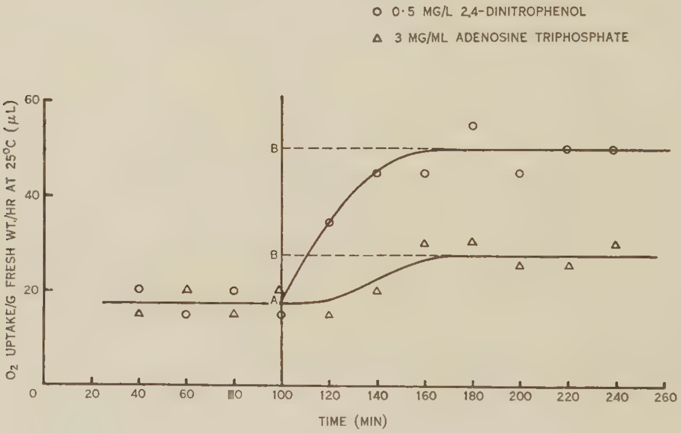


Fig. 3.—Results of a typical experiment to measure cut-tissue respiration rate, indicating the method of obtaining the results.

The contents of the side-arm were added to the disks which had been respiring for about 2 hr in the Warburg vessels. Figure 3 shows results of a typical experiment. These experiments were used for calculating the points shown in Figure 4[*]. The respiration rate at the time of adding ATP or DNP (point *A*, Fig. 3) was subtracted from the rate at 80 min after adding the substance to be tested (point *B*) and the difference calculated as a percentage of the rate before adding the substance. Figure 4 represents the percentage increase in respiration rate produced after adding the contents of the side-arm.

[*] The scatter of points is due to high variability between fruits, which influences all respiration results. Samples large enough to overcome this are impracticable to handle.

In the very early samples of the 1952-53 season up to 60 days from full blossom, the effect of adding DNP was small. By 130 days, the DNP had caused about 75 per cent. increase in respiration rate. The increased respiration rate then decreased slowly to 226 days. The percentage increase in respiration rate due to DNP was high at 130 days from blossom in the 1951-52 season also, and low at the end of the season when the whole-fruit respiration was rising. In both seasons stimulation of respiration was marked at about 150 days and its extent decreased as the fruit approached the climacteric rise.

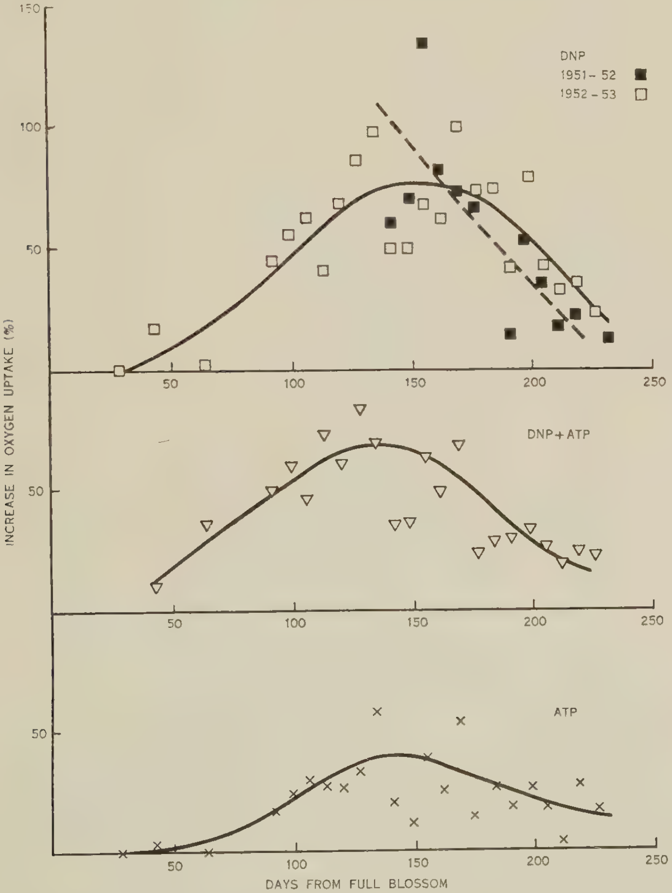


Fig. 4.—Percentage increase in respiration rate of cut tissue after adding 2,4-dinitrophenol, adenosine triphosphate, and both together (DNP, 0.5 mg/l; ATP, 3 mg/ml) at different stages of development.

(iii) *Action of Adenosine Triphosphate.*—In the 1952-53 season adenosine triphosphate (ATP) was added to give 3 mg/ml in contact with the disks (Fig. 4). ATP had no action in the early stages of development but increased the respiratory level by about 35 per cent. at about 150 days. The effect of ATP decreased at the end of the season.

(iv) *Action of ATP and DNP Added Together.*—In the 1952-53 season, ATP (3 mg/ml) and DNP (0.5 mg/l) were added to the disks at the same time. The results of this treatment are given in Figure 4. The curve is similar to that for DNP.

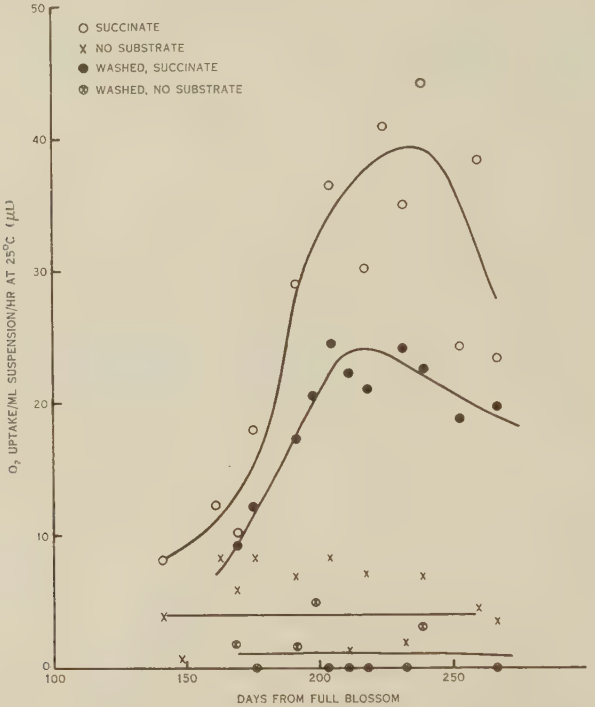


Fig. 5.—Oxygen uptake of cell-free particles prepared in water before and after washing, with and without substrates. Complete system in Warburg vessel: 1 ml particle suspension, 0.05M sodium succinate, and approx. 1×10^{-4}M cytochrome *c*. Temp. 27°C.

(*c*) *Succinoxidase Activity of Isolated Particles*

The oxidative activity (unwashed residue prepared in water) in the presence of 0.05M sodium succinate and in the absence of substrate is shown for the 1951-52 season in Figure 5. The activity, given as O_2 uptake per ml suspension per hour, is plotted against days from blossom. The activity increased sharply and then decreased towards the end of the season. The washed residue in 1951-52 (Fig. 5) showed lower activity both in the presence and absence of substrate but the trend was the same with an increase followed by a slight decrease towards the end of the season. The unwashed residue gave greater oxygen uptake both with and without substrate than the washed residue and was also more variable, probably because of the different amounts of substrate carried over.

The oxidative activity of unwashed residue prepared in sucrose in the 1952-53 season was determined. The results in the presence of 0.05M sodium succinate and in the absence of substrate are shown in Figure 6. The results were very similar to those for the water preparation in the same season. Unfortunately no samples were prepared in sucrose before 64 days from blossom.

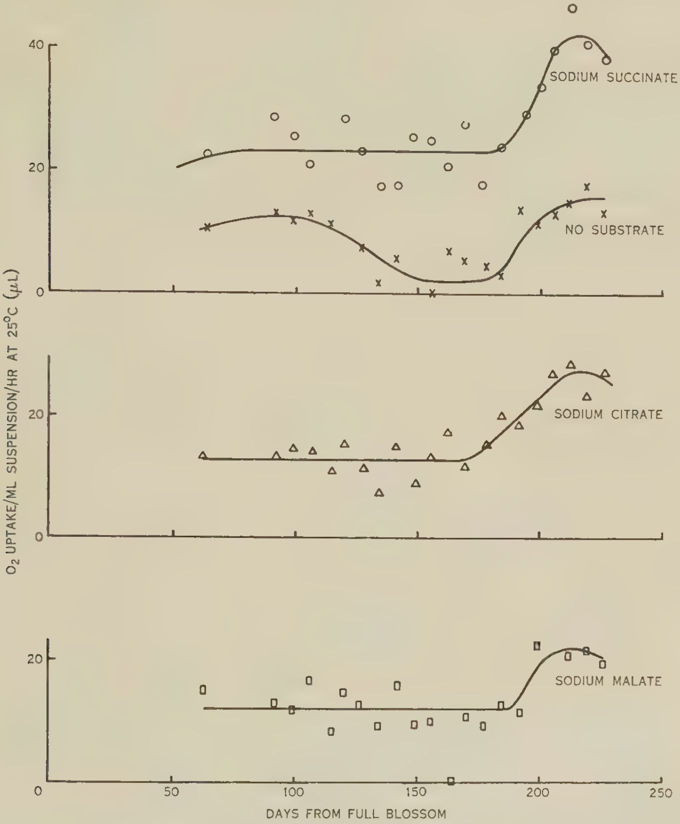


Fig. 6.—Oxygen uptake of cell-free particles (prepared in sucrose) with sodium succinate, sodium citrate, sodium malate, and no substrate. Complete system in Warburg vessel: 1 ml particle suspension, 0.05M sodium succinate, 0.02M sodium citrate or 0.02M sodium malate, 0.001M $MgSO_4$, 0.01M phosphate buffer pH 7, 0.001M adenosine triphosphate, and 0.3M sucrose. Temp. 25°C.

Samples prepared in water during this period gave a much higher activity per unit weight of original tissue, indicative of the greater concentration of active material in the small unvacuolated cells. From 70 to 190 days the activity was at the same level as the water preparation but the fluctuations were less in sucrose. At 190 days the respiration rate rose to a higher value at about 215 days. The activity in the absence of substrate was mostly higher in sucrose than in water. In sucrose the curve for oxygen uptake without substrate was very similar to that with substrate and a considerable increase in activity occurred at 190 days.

The marked increase in extractable succinoxidase activity occurring in all these experiments takes place about the same time as the respiration of the whole fruit begins to rise in the climacteric.

(*d*) *Citrate Oxidation by Isolated Particles*

The oxidative activity of isolated particles prepared in sucrose was measured in the presence of 0.02M sodium citrate (Fig. 6). The rate is about half the average rate with succinate up to about 170 days. The increase in activity occurred earlier and by 210 days the rate had doubled. The activity of the water preparation was also measured with 0.02M sodium citrate but by 150 days the activity was less than in the sucrose preparation and the experiments on the water preparations were not continued.

(*e*) *Malate Oxidation by Isolated Particles*

The oxidative activity of isolated particles prepared in sucrose was measured in the presence of 0.02M sodium malate. The results (Fig. 6) show a level of activity slightly less than in citrate with a sharp rise occurring at 197 days, which doubled the rate.

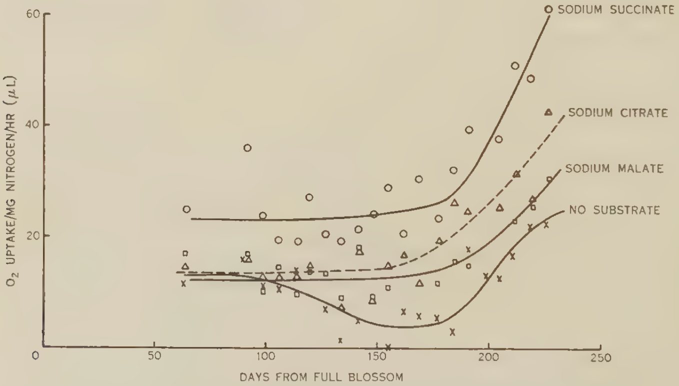


Fig. 7.—Oxygen uptake of cell-free particles prepared in sucrose and expressed per mg nitrogen in the preparation.

(*f*) *Activity of Isolated Particles*

The total nitrogen of the residue was estimated in each sample and the activity of the residue with succinate, citrate, and malate was expressed per mg nitrogen (Fig. 7). The curves seem to show little change until the sharp rise at 190 days which continued to 226 days when the last sample was taken. The oxygen uptake per mg nitrogen in the absence of substrate seemed to decrease towards 150 days but showed the sharp increase at about 190 days. The decrease in activity in the absence of added substrate between 100 and 150 days may be a reflection of the decrease in concentration of organic acids in the tissue, which would be the substrates carried over during the preparation.

Millerd, Bonner, and Biale (1953) extracted particles from the avocado at higher speeds (two successive spins of 16,000g for 15 min) after a preliminary spin to separate the larger particles such as nuclei. An apple-particle preparation made in the same way gave an activity per mg N which did not differ significantly from that of our standard method; the activity was only about one-tenth that reported by Millerd, Bonner, and Biale for the avocado particles.

The definite utilization of succinic acid by the particles suggests the activity of a cytochrome-cytochrome oxidase system. The utilization of succinate, citrate, and malate by these particles provides evidence for the possible participation of a tricarboxylic acid cycle.

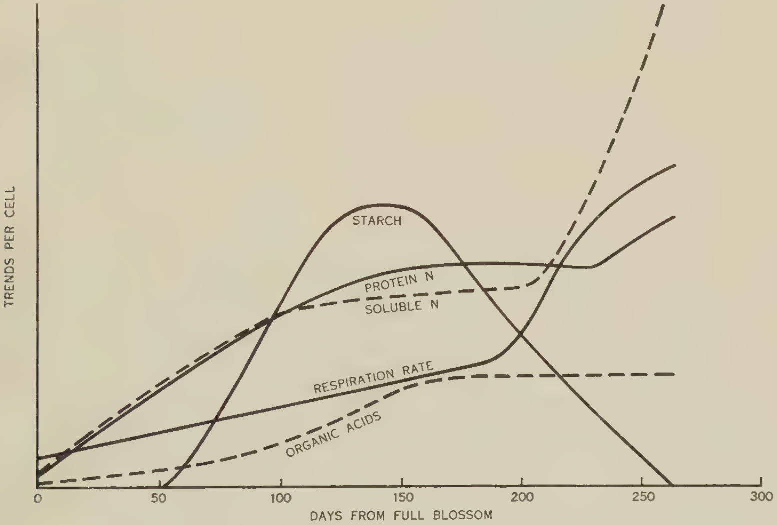
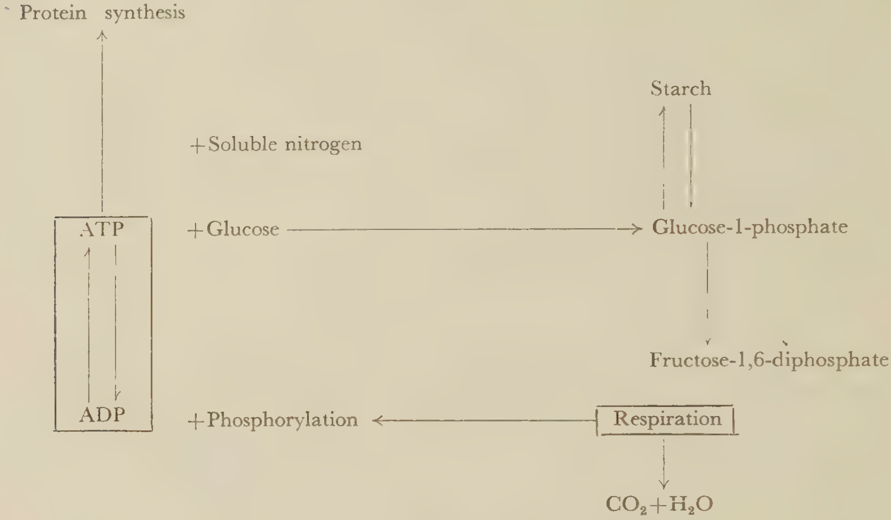


Fig. 8.—Generalized curves based on earlier work on apples (Pearson and Robertson 1953) to show the relations in time between the changes in starch, protein and soluble nitrogen, organic acids, and respiration rate.

IV. Discussion

It has been shown in earlier work on fruit from some of the same trees (Robertson and Turner 1951; Pearson and Robertson 1953) that a number of changes took place in the cells prior to and during the climacteric rise (Fig. 8). Protein, soluble nitrogen compounds, and starch seem to be involved. Starch content approached its maximum at about 120 days from full blossom and decreased from about 165 days onwards. At about the same time, protein and soluble nitrogen, which had been increasing in the cells, stopped increasing for some weeks and then decreased slightly. At about 200 days, just after the onset of the climacteric rise, the soluble nitrogen increased very rapidly in the cells and, after some delay, protein nitrogen began to increase again. These processes as they occurred in 1949, 1950, and 1951, are represented diagrammatically in Figure 8.

The interrelations of starch, protein and soluble nitrogen, and respiration rate can be shown as follows:



The respiration rate increased in the climacteric rise at about 190 days from full blossom and continued to rise while the fruit remained on the tree (Fig. 1). The respiration rate per unit weight of cut tissue fell rapidly till 130 days and then remained at about the same rate till the end of the season. When, however, cut-tissue and whole-fruit respiration are compared on a per-fruit basis (equivalent to a per-cell basis), they are very similar in young fruits (Fig. 2). The cut-tissue rate increases more rapidly than the whole-fruit rate, which remains steady from about 100 to 190 days. At 190 days both whole-fruit respiration and cut-tissue respiration begin to rise and this continues until the end of the observations. Similar trends were seen in both seasons. Millerd, Bonner, and Biale (1953) noted that the climacteric rise was not as marked in slices of avocado as in the intact fruit and that the difference in rates was more marked in pre-climacteric fruit than in climacteric fruit. The figures tabulated by these authors show no climacteric rise in respiration of cut tissue.

(*a*) *Control of Respiration Rate*

The rate of respiration in these experiments has been increased in four separate ways: (1) by the climacteric rise, (2) by cutting, (3) by the addition of DNP, and (4) by the addition of ATP. Diagrammatic respiration curves taken from Figures 2 and 4 are compared in Figure 9.

According to the hypothesis outlined in the introduction, one of the most important factors controlling the respiration rate is the phosphate carrier system. If it can be assumed that, as in other tissues, this is adenosine polyphosphate, then the concentration of ADP will govern the rate. This is inversely related to the ATP/ADP ratio at a constant concentration of adenosine polyphosphate. The respiration rate will be low when this ratio is high because the ADP concentration will then be low, and will increase, with increase in the ADP to accept phosphate, when this ratio falls. It has been suggested (Pearson and Robertson 1953) that the sharp increase in respiration rate (Fig. 8) at about 190 days from blossom is due to the increase in ADP relative to ATP when the maintenance of protein and other unstable compounds already synthesized makes high demands on the energy-carrying system.

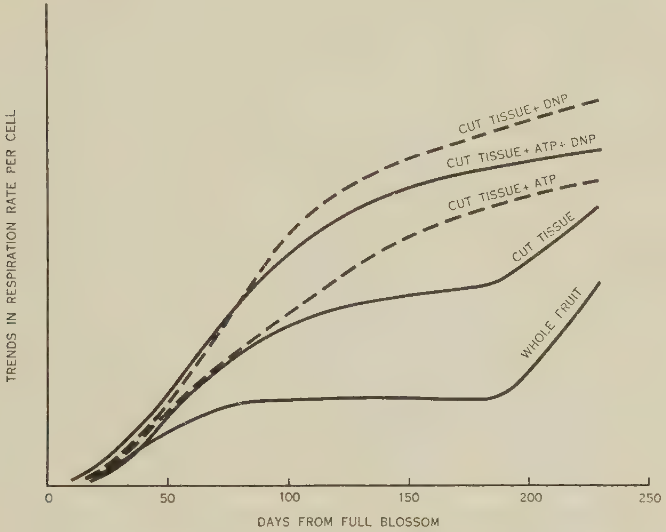


Fig. 9.—Generalized curves for respiration rate per cell based on the changes in 1952-53 season (see Fig. 2).

The increase in respiration rate on cutting (Figs. 1, 2), which occurs between 50 days and about 230 days from blossom, is not inconsistent with the hypothesis. Many workers have observed that mechanical stimulus and wounding both increase the respiration of plant tissue. The greater rate observed in cut tissue may be stimulated by the shearing and squeezing effects on cells not actually cut. At present it is impossible to say by what means this stimulus increases the rate, since it might affect a number of factors. From the hypothesis, however, any effect of cutting likely to lower the ATP/ADP ratio would raise the respiration rate. Such factors would be the increased demands of synthesis as the cells adapted themselves after cutting or increase in the activity of an ATP-splitting enzyme which had been liberated. Millerd, Bonner, and Biale (1953) have suggested that the ATP-ase of mitochondria might take part in the ripening of the avocado.

Whatever the cause of the increased respiration in the cut tissue, it seems that the respiration rate is still controlled to some extent by the ATP/ADP ratio. Up to 65 days from blossom, addition of DNP to cut tissue had very little effect (Figs. 2, 4); thereafter, prior to the climacteric, there was a marked increase due to DNP in the oxygen uptake. The percentage stimulation due to DNP gradually decreased after about 160 days and was at a minimum after the rise in the whole fruit respiration and in the control cut tissue (Fig. 4). Examination of Figure 9 shows that this decrease in percentage stimulation is due to the increase in cut-tissue respiration, which accompanies the rise in the whole-fruit respiration. These results we interpret as being due to the controlling effect of the ATP/ADP ratio in cut tissue from about 65 days until the climacteric rise. After 65 days, the ratio in cut tissue might have been lower than that in whole-fruit tissue, thus explaining the increase in respiration rate on cutting, which, however, was not as effective in increasing oxygen uptake as the uncoupling effect of the DNP. As the fruits went through the climacteric rise, the oxygen uptake per cell of cut tissue increased too, but the oxygen uptake per cell in the presence of DNP increased only slightly with increase in cell size, i.e. showed no climacteric rise. This suggests that the ATP/ADP ratio remains in control of the respiration at all stages but a change in the ratio allows an increase in both whole-fruit and cut-tissue respiration after 190 days.

The hypothesis that the ADP concentration limits the rate of respiration in fruit from about 65 to 190 days is further supported by the results with added ATP. The hypothesis that the ATP/ADP ratio controls the respiration rate is based on the assumption that the amount of adenosine polyphosphate which occurs naturally in the cells does not alter rapidly. If, however, the absolute amount is increased by the absorption of ATP supplied to the external medium, the amount of ADP will be increased even though the ratio remains the same. This will result in increased respiration rate. No stimulation by ATP addition was obtained until after 65 days. ATP then influenced the oxygen uptake with a trend similar to that shown by the addition of DNP (Fig. 4). As the fruit approached the climacteric rise, the percentage stimulation due to added ATP decreased. As seen in Figure 9, this decrease in percentage is associated with the increase in cut-tissue respiration.

The simultaneous addition of ATP and DNP gave a curve similar to that obtained with DNP alone. The fact that the stimulation due to DNP added simultaneously with ATP was not greater than that due to DNP alone indicated that the uncoupling of respiration from phosphorylation by DNP was complete, since addition of more phosphate carrier did not increase oxygen uptake. No suggestion is offered to explain why the respiration with DNP and ATP was less than that with DNP alone.

The respiration rate is thus interpreted as being controlled by the ATP/ADP ratio, a conclusion similar to that reached by Millerd, Bonner, and Biale (1953) for the avocado. Our hypothesis given earlier (Pearson and Robertson 1953) differs from theirs in attributing the change in respiration rate, at the time of the climacteric rise, to the increased demands of synthetic processes to maintain the protein content of the cells. Millerd, Bonner, and Biale attribute the change to an increase in a natural uncoupling agent which behaves like DNP. This might be ATP-ase. There is at present insufficient evidence to decide between these two hypotheses and it is not necessarily true that the change in ATP/ADP ratio would be due to the same cause in a fruit like an apple developing slowly on a tree and a fruit like an avocado with a sharp ripening phase. The evidence for our hypothesis for the apple is discussed further in the next section.

(*b*) *Control of Synthesis*

Using the hypothesis to examine the results of several seasons, we can suggest how synthesis is related to respiration rate during development (see Fig. 8). In the early stages of development up to about 65 days, when the DNP and ATP have very little effect on the respiration, the ATP/ADP ratio can be interpreted as being low. The respiration rate per unit weight at this time is high, though the respiration per cell is low because of the small cell size. The active synthesis of protein and other compounds necessary for the growth of the cell uses the ATP formed in this period. After about 60 days from blossom, starch begins to appear and increases steadily till about 120 days and then decreases from about 165 days. During the period of starch increase and maintenance, the ATP/ADP ratio is probably high and the phosphorylations, in excess of those required for growth, result in the formation of the glucose-1-phosphate, which is condensed to starch. This corresponds to the period during which the respiration is controlled and the stimulation with both DNP and ATP rises to its maximum.

Protein nitrogen increases steadily through the early part of the season but the rate of increase falls off at about the time of the starch maximum. It is possible that the protein increase is checked by the decreased rate of import of soluble nitrogen which occurs at about the 100th day. It is significant, however, that McKee and Urbach (1953) have shown that a marked decrease in the content of glutamine occurs at about this time and that no glutamine could be detected at 138 days in spite of no marked change in glutamic acid. This they point out may be due to insufficient ATP for this reaction. ATP is known to be required for glutamine synthesis *in vitro*. At this time, starch and protein content, both dependent on ATP, are being maintained but by the 165th day the synthesis of starch does not exceed its rate of utilization and this can be taken as further indication that the ATP/ADP ratio is beginning to decrease. This may be due to the protein maintenance, which has increased with increase in cell size, competing with the other processes requiring phosphorylation; the transfer of energy is sufficient only for the resynthesis of the unstable protein and of other compounds in the cells by that time. By 190 days, the starch content has decreased and the decreasing ATP/ADP ratio is shown by the increase in respiration rate. The adjustment of the respiration rate to the ATP/ADP ratio is not continuous but is marked by a sudden rise; kinetic considerations of the hypothesis show that the respiration rate is not an inverse linear function of the ratio, but that the lower the ratio the more rapidly does the respiration increase for a given change of ratio. Even after the respiration rate has increased no net gain of starch is possible and there is no change in the protein nitrogen concentration until about 230 days, despite the fact that the soluble nitrogen has begun its rapid increase at 200 days. When the protein nitrogen does increase again towards the end of the life of the fruit on the tree, only a relatively small fraction of the soluble nitrogen is changed. This increase in protein nitrogen, however, indicates that there is some ATP available for net gain at the higher respiration rate, in addition to the ATP required for maintenance. The fact that the respiration is not completely uncoupled from phosphorylations at this stage is also suggested by the stimulatory effect of DNP which, though small, is still apparent at the end of the observations. When the DNP effect has reached its minimum, probably corresponding to the lowest ATP/ADP ratio since the early stages of development (Fig. 4), the spectacular increase in respiration occurs. This increased respiration, after a time, results in the gain in total protein late in the life of the fruit, which uses only a small fraction of the increased soluble nitrogen.

During the period from 60 to 190 days, very little change in the activity of the particles with substrates of succinate, citrate, or malate was observed. When the respiration of whole tissue increased in the climacteric rise, the activity of the particles also increased (Figs. 5, 6). Increased activity per mg of nitrogen in the particles (Fig. 7) implies either that the oxygen uptake by the particles had been controlled by some factor such as the ATP/ADP ratio in the particles, which changed at the time of the climacteric, or that the enzyme concentrations had increased relative to the other nitrogen constituents. The second possibility would seem to be less likely than the first since increase in the concentrations of the oxidative enzymes would be expected to cause a sharp rise in the DNP respiration of cut tissue, but this did not occur (Fig. 4). Control of oxidation rate by ATP/ADP in the extracted particles is consistent with the general hypothesis of control in the intact tissue. In this respect, the apple differs from the avocado investigated by Millerd, Bonner, and Biale, who found no evidence of a change in the activity of isolated particles in pre-climacteric and post-climacteric fruits. This might be a consequence of the differences between a developing fruit and a detached fruit in which the climacteric takes place rapidly.

While it has been postulated that in apples between 65 and 190 days the respiration rate is limited by the ATP/ADP ratio, once the climacteric rise starts at least two factors will contribute to the rise continuing. The breakdown of starch which is taking place concurrently in these fruits will contribute a phosphorylated hexose to enter the respiration system and hence act as a source of substrate in the cytoplasm, so that substrate supply is less likely to become limiting. In this connection, it is important to note that the loss of starch between 185 and 225 days (see Pearson and Robertson 1953) is about 8×10^{-8} g per cell or 0.2×10^{-8} g per cell per day. If all this starch were respired as phosphorylated hexose it would produce 1.4×10^{-7} mg CO_2/cell/hr, and as the respiration rate at 225 days is 1.2×10^{-7} mg CO_2/hr, the hexose from starch would account approximately for the increase in respiration rate.

The other factor is the increase in respiratory enzyme activity which suggests that the enzyme systems can keep pace with the increased rate of respiration. In the earlier paper, it was observed that the total organic acid content did not change (Fig. 8) at the time of the climacteric rise. It has now been shown that the activity of the enzymes concerned with the acid cycle had doubled within 36 days of the onset of the climacteric (Fig. 7). The absence of change in the organic acid content at this time indicates either that the gross concentration does not relate very directly to the respiratory activity or that there is an increased rate of turnover in acid without any change in net content.

The occurrence of the appropriate enzymes in the particles is further evidence for the participation of the tricarboxylic acid cycle in the respiration but the cause of the accumulation of organic acids, chiefly malic, which may be in the vacuole, requires more investigation.

Much has been written on the climacteric rise in apples and some of the problems have been discussed at length by Kidd and West (1945). The hypothesis outlined in this paper may serve as a starting point for re-examination of the theoretical implications of results given by Kidd and West in their various publications. Such factors as the influence of ethylene and the effect of time of picking on the subsequent climacteric require explanation. While we do not wish to attempt this in the present paper, it seems likely that the hypothesis we discuss will explain the "auto-regulatory mechanism" controlling respiration during growth suggested by Kidd and West.

(*c*) *Conclusion*

Thus it may be concluded that the hypothesis connecting respiration rate with starch, organic acid, and protein metabolism through the ATP/ADP ratio is consistent with our present knowledge. The climacteric rise in respiration is due primarily to increase in ADP concentration with the change in this ratio and to the tissue's capacity for increasing the activity of respiratory enzymes. It is hoped that our hypothesis can be further examined when it becomes possible to estimate the ATP/ADP ratio directly.

V. ACKNOWLEDGMENTS

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THE BREAKDOWN OF CARBOHYDRATES BY *ASTEROCOCCUS MYCOIDES*, THE ORGANISM OF BOVINE PLEUROPNEUMONIA

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Summary

Cell suspensions of *A. mycoides* oxidized glucose, fructose, and mannose. Glucose, pyruvate, lactate, and, in the presence of catalase, glycerol were oxidized quantitatively to acetate and carbon dioxide. Anaerobically, pyruvate underwent dismutation to acetate, lactate, and carbon dioxide. Neither glucose nor glycerol were attacked anaerobically by intact cell suspensions. No other pentoses, hexoses, di-, tri-, or polysaccharides, or polyhydric alcohols, were attacked by cells grown in a complex undefined medium.

None of the following substances was attacked either by intact cell suspensions or by suspensions treated by freezing and thawing: formate, acetate, succinate, fumarate, malate, citrate, aspartate, glutamate, α-ketoglutarate, alanine, or proline. It is concluded that the organism possesses no Wood-Werkman system, or di- or tricarboxylic acid cycle for the oxidation of these substances.

Evidence was obtained that the organism lacks a cytochrome system, but possesses catalase. Growth in a complex medium was almost absent under anaerobic conditions and was greatly increased by aeration, but although strongly aerobic in its growth requirements, *A. mycoides* resembles in its metabolism the facultative organisms rather than the strict aerobes.

I. Introduction

The organism of bovine pleuropneumonia (*Asterococcus mycoides*) and the "pleuropneumonia-like" organisms isolated from other sources, e.g. from mice, human and bovine genital tracts, sheep, goats, and chick embryo, form a group of closely related species possessing many similarities to the L forms of bacteria (Tulasne 1951; Dienes and Weinberger 1951).

Virtually nothing, however, has yet been published concerning the intermediary metabolism of any of the organisms belonging to this group, or of bacteria in the L phase. Dujardin-Beaumetz (1900) identified acetic acid as the sole volatile acid produced in cultures of *A. mycoides* in media containing fermentable carbohydrate. Holmes and Pirie (1932) showed the presence of lactic dehydrogenase in resting cell suspensions, but did not obtain methylene blue reduction with glucose, formate, succinate, alanine, or hypoxanthine as substrates. Similar results were reported by Warren (1942). Recently, Somerson and Morton (1953) have interpreted the accumulation of formazan deriva-

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tives in surface colonies of pleuropneumonia-like organisms on media containing tetrazolium salts as evidence for the presence of flavoprotein oxidases.

In this paper, the results of a general survey of the overall catabolic reactions catalysed by *A. mycoides* are described. It is hoped later to extend the observations to include some L forms of bacteria and their parent organisms.

II. Methods and Materials

(*a*) *Cultural Methods*

(i) *The Organism.*—Strain "V5" was isolated from a case of acute bovine contagious pleuropneumonia in 1936, and has since been used for the preparation of prophylactic vaccine (Campbell 1938).

(ii) *Medium.*—Cells were harvested from cultures in BVF-OS medium (Turner, Campbell, and Dick 1935). It is essentially a pig-stomach digest of beef liver and muscle, buffered with M/15 phosphate at pH 7.4. Ox serum (10 per cent. v/v) is added before sterilizing the medium by filtration through Seitz pads.

(iii) *Growth Conditions.*—Cells were grown on 2 l. of medium in 5-l. florence flasks fitted with rubber bungs through which passed a filling and inoculating tube and a second tube terminating in a sintered glass disk of porosity 4. On inoculating with 2 ml of a 3-4 day culture, oxygen containing 5 per cent. carbon dioxide was passed through the sintered glass disk at an approximate rate of 150 ml/hr during incubation at 37°C for 40 hr. An electrically heated nichrome wire in the neck of the flask prevented froth reaching the cotton-wool plugs.

Cultures grown under these conditions consisted mainly of small forms appearing as points of light by microscopic examination with dark ground illumination, and occasional short filaments and discules.

(*b*) *Preparation and Standardization of Cell Suspensions*

(i) *Preparation of Suspensions.*—The cells were recovered from the cultures by passage through a steam-driven Sharples supercentrifuge (62,400g at periphery of cylinder). The cylinder was lined with a plastic parchment-like material, from which the cell material was scraped off and resuspended in distilled water. The cells were not washed, since endogenous respiration of unwashed cells during the usual manometric experiments was low, and washing was found to decrease the rate of oxidation of all substrates tested. Cell suspensions were used within 6 hr of their preparation. The activity of suspensions was not increased, nor the loss of activity after storage decreased, by suspending the cells in Krebs-Ringer solutions, saline, or buffers instead of distilled water.

(ii) *Standardization of Cell Suspensions.*—In most experiments optical density measurements were used, since it was found that, for suspensions prepared from cells grown under standard conditions, cell nitrogen and dry weight were related to the optical density of suspensions in water. In some experiments, the quantitative Biuret reaction (Stickland 1951) was used.

(iii) *Preparation of Frozen and Thawed Suspensions.*—Suspensions were rapidly frozen in a "dry-ice"-alcohol bath and allowed to thaw. The pH was maintained between 6.5 and 7.0 during thawing by the cautious addition of 0.05N sodium hydroxide. The process was repeated twice or thrice. The preparations became extremely mucoid during this treatment.

(iv) *Estimation of Growth in Cultures.*—Samples of the culture were centrifuged for 15 min at 15,000 r.p.m. in the high-speed head of the International centrifuge, the deposit evenly suspended in the same volume of water, and the dry weight determined from optical density measurements with a filter transmitting maximally at 440 mμ.

(*c*) *Analytical Methods*

(i) *Manometric Methods.*—Conventional Warburg manometers were used. Oxygen uptake as measured by the direct method of Warburg, and by the diethanolamine-carbon dioxide buffer method as modified by Krebs (1951) showed that the absence of carbon dioxide did not influence the oxygen uptake with glucose, lactate, or pyruvate as substrates. In all subsequent experiments the direct method of Warburg was used for measuring oxygen uptakes. Carbon dioxide evolution was measured after tipping in acid at the end of the experimental period. The experiments were carried out at a temperature of 37°C.

(ii) *Carbon Balance Experiments.*—Non-gaseous products were estimated in the contents of the manometer vessels. The contents of several flasks were pooled to obtain sufficient material for the estimations. Trichloracetic acid was used to deproteinize the material for pyruvate and lactic acid estimations. Samples for estimation of volatile acid and of acetoin (after oxidation to diacetyl) were steam-distilled in the Markham apparatus without previous deproteinization.

(iii) *Pyruvic Acid.*—Pyruvic acid was estimated by the method of Friedemann and Haugen (1943).

(iv) *Acetic Acid.*—Acetic acid was determined by the method of Friedemann (1938), and in some experiments, its identity was confirmed by partition chromatography on silica gel columns (Elsden 1946).

(v) *Lactic Acid.*—Lactic acid was estimated by the colorimetric method of Barker and Summerson as described by Umbreit, Burris, and Stauffer (1949).

(vi) *Acetoin.*—Acetoin was oxidized to diacetyl by excess ferric chloride (van Niel 1927), and estimated colorimetrically by the method of White, Krampitz, and Werkman (1946).

(*d*) *Materials*

(i) *Pyruvic Acid.*—Pyruvic acid was prepared by double distillation of commercial pyruvic acid as described by Lipschitz, Potter, and Elvehjem (1938), and used with the precautions concerning dilution and neutralization recommended by these authors.

(ii) *Coenzyme Concentrate.*—The mixed nucleotide "fraction *A*," prepared from fresh pig liver as described by Le Page and Mueller (1949) for the preparation of triphosphopyridine nucleotide, was used as a source of coenzymes. The preparation was assayed for coenzyme *A* activity by the method of Handschumacher, Mueller, and Strong (1951). Although not assayed for other coenzymes, similar preparations have been found to contain appreciable amounts of both di- and triphosphopyridine nucleotides, pyridoxal phosphate, cocarboxylase, and bound biotin, in addition to coenzyme *A* (Buyske *et al.* 1951).

(iii) *Catalase.*—Beef liver catalase was prepared by the method of Mosimann (1951).

(iv) *Diphosphopyridine Nucleotide.*—This was prepared by the method of Williamson and Green (1940), and its purity assayed enzymically with crystalline alcohol dehydrogenase prepared by the method of Racker (1950).

III. EXPERIMENTAL

(*a*) *Breakdown of Sugars, Polysaccharides, and Sugar Alcohols*

(i) *Substrates Attacked.*—The ability of cell suspensions to oxidize the sugars, polysaccharides, and sugar alcohols commonly used in determinative bacteriology was tested manometrically in phosphate buffer at pH 7.4. An oxygen uptake was found with glucose, mannose, and fructose. The rates, expressed as Q_{O_2} (N) for these three hexoses with suspension from the same batch of cells, were 350, 330, and 130 respectively. Glycerol was attacked at a high initial rate, but the oxygen uptake had almost ceased within 15 min of adding the substrate. In the presence of catalase, oxygen uptake continued at a constant rate corresponding to a Q_{O_2} (N) of 360 (Fig. 1). No other pentoses, di-, tri-, or polysaccharides, or sugar alcohols tested were attacked by cells grown in BVF-OS medium. The pH had little effect on the rate of glucose oxidation over the range 7-8; the rate decreased to about half this value at a pH of 8.8.

(ii) *Fermentation of Glucose and Glycerol.*—The ability of cell suspensions to form acid from glucose and glycerol was tested in bicarbonate buffer with 5 per cent. carbon dioxide in nitrogen as the gas phase. None was formed from either substrate, although the suspension oxidized glucose vigorously when tested aerobically in phosphate buffer. The formation of lactic acid from glucose anaerobically was also tested in phosphate buffer with oxygen-free nitrogen as the gas phase. Lactic acid was estimated in the flask contents after 60 min incubation. Lactic acid formation was negligible and less than in the control flask without glucose.

(iii) *Products of Glucose Oxidation.*—The results obtained in four separate experiments for the oxidation of glucose by cell suspensions in phosphate buffer are shown in Table 1. Acetate accumulated as the end-product of the oxidation. Carbon recovery was complete. The figures agree with the equation for the reaction:

$$C_6H_{12}O_6 + 2O_2 \longrightarrow 2CO_2 + 2CH_3COOH + 2H_2O.$$

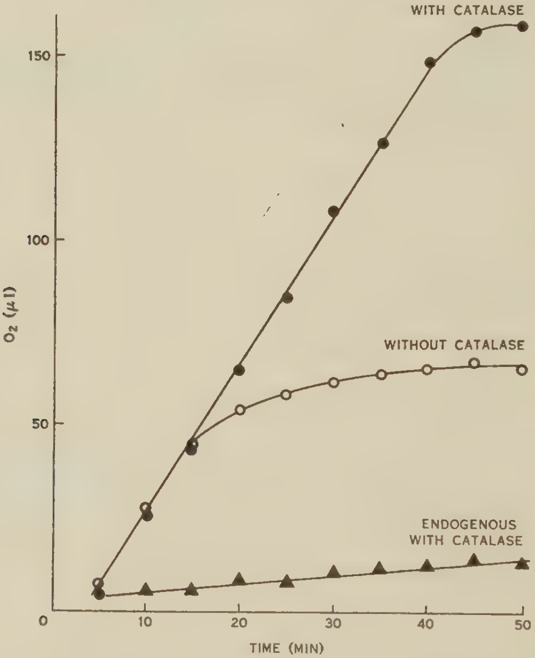

Fig. 1.—Effect of catalase on glycerol oxidation by suspensions of *A. mycoides*. Warburg vessels contained: M/5 phosphate buffer, 1.45 ml; bacterial suspension, 0.5 ml ≡ 0.68 mg N (main compartment); catalase solution, 0.25 ml (side bulb 1); M/20 glycerol, 0.1 ml (side bulb 2); 20 per cent. w/v KOH, 0.2 ml (centre well).

(iv) *Products of Glycerol Oxidation.*—The products of the oxidation of glycerol in the presence of catalase are shown in Table 2. Carbon recovery in the products was again complete, and the figures agree with the equation:

$$C_3H_8O_3 + 1.5O_2 \longrightarrow CO_2 + CH_3COOH + 2H_2O.$$

(*b*) *Breakdown of Pyruvate and Lactate*

(i) *Pyruvate Oxidation.*—The cell membrane of the organism appears to be permeable to pyruvate, since it was oxidized at a rate comparable with that found with other substrates, and the rate of oxygen uptake did not differ over a range of pyruvate concentrations of 0.5-0.01M. Lower pyruvate concentrations were not tested.

TABLE 1

PRODUCTS OF GLUCOSE OXIDATION BY *A. MYCOIDES*

Warburg vessels contained: M/5 phosphate buffer (pH 7·4) 1·5 ml; bacterial suspension 0·5 ml; M/40 glucose 0·2 ml (5μM)

Values in μM

Experiment	Glucose Oxidized	Oxygen	Carbon Dioxide	Acetate
1	5	9·8	9·8	9·65
2	5	9·4	10·3	11·35
3	5	9·95	10·9	8·4
4	5	8·75	9·9	10·9
Mean	5	9·5	10·25	10·1
mg C	0·36		0·123	0·242

% C recovered = 100

The products for pyruvate oxidation are shown in Table 3. The figures agree with the equation:

$$CH_3CO.COOH + \frac{1}{2}O_2 \longrightarrow CO_2 + CH_3COOH.$$

TABLE 2

PRODUCTS OF GLYCEROL OXIDATION BY *A. MYCOIDES*

Warburg vessels contained: M/5 phosphate buffer (pH 7·4) 1·2 ml; bacterial suspension 1·0 ml; catalase solution 0·3 ml; M/20 glycerol 0·2 ml (10μM)

Values in μM

	Glycerol Oxidized	Oxygen	Carbon Dioxide	Acetate
	10	14·3	9·2	10·0
mg C	0·36		0·110	0·240

% C recovered = 97

(ii) *Pyruvate Dismutation.*—Pyruvate was also attacked anaerobically. The results obtained in two separate experiments for the anaerobic breakdown of pyruvate in phosphate buffer, with oxygen-free nitrogen as the gas phase, are shown in Table 4. Recovery of carbon in the products in these experiments was only 87 per cent. Lactate and acetate were found in approximately equimolar amounts, and carbon dioxide in slightly greater amounts. The figures are consistent with the breakdown of pyruvate by the dismutation reaction, together with the formation of a small amount of acetoin:

$$2CH_3CO.COOH + H_2O \longrightarrow CH_3COOH + CH_3CHOH.COOH + CO_2$$

$$2CH_3CO.COOH \longrightarrow CH_3CHOH.COCH_3 + 2CO_2.$$

The formation of small amounts of acetoin during the dismutation reaction was shown subsequently.

Table 3

PRODUCTS OF PYRUVATE OXIDATION BY *A. MYCOIDES*

Warburg vessels contained: M/5 phosphate buffer (pH 7·4) 1·0 ml; bacterial suspension 0·4 ml; M/10 pyruvate 0·2 ml (20μM)

Values in μM

Pyruvate Oxidized	Oxygen	Carbon Dioxide	Acetate
20	9·1	18·0	19·4
mg C 0·72		0·216	0·466

% C recovered = 95

Table 4

PRODUCTS OF ANAEROBIC BREAKDOWN OF PYRUVATE BY *A. MYCOIDES*

Warburg vessels contained: M/5 phosphate buffer (pH 7·4) 1·25 ml; M/10 pyruvate 0·2 ml (20μM); bacterial suspension 0·5 ml

Values in μM

Experiment	Pyruvate Disappearing	Carbon Dioxide	Lactate	Acetate
1	20	10·0	8·06	8·34
2	20	9·9	9·02	8·42
Mean	20	9·95	8·54	8·38
mg C	0·72	0·12	0·308	0·200

% C recovered = 87

(iii) *Oxidation of Lactate.*—Methylene blue reduction was relatively rapid in Thunberg tube experiments with lactate as substrate compared with other substrates tested. In one experiment, for example, the times for 90 per cent. decolourization were 1, 7, and 15 min with lactate, glucose, and pyruvate respectively. The products for the oxidation of DL-lactate are shown in Table 5. The figures agree with the equation:

$$CH_3CHOH.COOH + O_2 \longrightarrow CH_3COOH + CO_2 + H_2O.$$

Slightly less than half was oxidized, whereas a sample of L-lactate was wholly oxidized.

(*c*) *Oxidation of Tricarboxylic Acid Cycle Intermediates*

The ability of the organism to metabolize some of the intermediate compounds of the tricarboxylic acid cycle, and also the amino acids which may be converted by deamination or transamination to tricarboxylic acid cycle intermediates, was tested both in Thunberg tube experiments with methylene blue as electron acceptor, and in manometric experiments in which oxygen uptake was measured. Intact cell suspensions, as well as suspensions treated by freezing and thawing to break down permeability barriers, were tested. The following substances were not attacked in any of these experiments: citrate, malate, succinate, fumarate, formate, acetate, glutamate, aspartate, α-ketoglutarate, proline, or alanine. The ability of suspensions treated by freezing and thawing to oxidize acetate, malate, citrate, succinate, and glutamate was also tested manometrically in bicarbonate buffer with 5 per cent. carbon dioxide in nitrogen as the gas phase. Methylene blue, which can replace oxygen as electron acceptor for the oxidation of glucose, was added as electron acceptor in this experiment. The pig-liver preparation "fraction *A*" was used as a source of coenzymes. The manometer vessels contained 1.25μM of glucose, or 2.5μM of the other substrates, 8μM methylene blue, 1 mg of the "fraction *A*" preparation, and suspension equivalent to 5 mg nitrogen. The evolution of carbon dioxide from glucose was very rapid and was almost complete within 45 min, whereas with the other substances it was very slow and was the same as that without substrate.

TABLE 5

PRODUCTS OF LACTATE OXIDATION BY *A. MYCOIDES*

Warburg vessels contained: M/5 phosphate buffer (pH 7·4) 1.5 ml; bacterial suspension 0·5 ml; M/10 DL-lactate 0·2 ml (20μM)

Values in μM

Initial Lactate	Final Lactate	Lactate Oxidized	Oxygen	Carbon Dioxide	Acetate
20	11·2	8·8	8·54	8·54	8·55
mg C		0·317		0·102	0·205

% C recovered = 97

(*d*) *Terminal Respiratory Enzymes*

(i) *Cytochrome System.*—It was concluded from the following evidence that the organism lacks a cytochrome system.

(1) The rate of glucose oxidation was not very sensitive to inhibition by cyanide, a concentration of 0.46×10^{-3}M caused only a 30 per cent. inhibition of the rate. Pyruvate oxidation was relatively insensitive to inhibition by azide; concentrations of 10^{-2}M and 10^{-3}M inhibited the rate of oxygen uptake by 60 per cent. and 20 per cent. respectively.

(2) Cytochrome bands could not be detected upon spectroscopic examination, even after freezing in liquid air.

(3) Cell suspensions were incapable of oxidizing *p*-phenylene diamine when tested manometrically.

TABLE 6

GASEOUS REQUIREMENTS FOR GROWTH OF *A. MYCOIDES* IN BVF-OS MEDIUM

50-ml volumes of medium in 250-ml erlenmeyer flasks; liquid depth approximately 1 cm. For deep culture 50 ml medium in narrow bottle; liquid depth approximately 7 cm. Inoculum: 0·02 ml culture. — = No detectable growth

Gas Phase	Growth (mg dry wt./ml culture)	
	40 Hr	64 Hr
H_2	—	0·05
N_2	—	0·075
O_2	—	0·063
38 mm CO_2; 722 mm H_2	—	0·14
44 mm CO_2; 5 mm O_2; 711 mm N_2	0·045	0·215
53 mm CO_2; 140 mm O_2; 567 mm N_2	0·19	0·292
40 mm CO_2; 720 mm O_2	0·225	0·425
Air (liquid depth 1 cm)	0·115	0·385
Air (liquid depth 7 cm)	0·06	0·135

(ii) *Gaseous Requirements for Growth in BVF-OS Medium.*—To test the effect of the oxygen and carbon dioxide tensions in the gas phase on growth of the organism in BVF-OS medium, 250-ml erlenmeyer flasks containing 50-ml volumes of medium were inoculated with 0.02 ml of culture. The flasks were incubated in closed jars which were evacuated and filled (twice) with the appropriate gas mixture. In addition one flask was exposed to the air of the incubator, and also one narrow bottle containing the same volume of medium, but having a liquid depth of approximately 7 cm. Residual oxygen in the gas phase of the jars containing hydrogen or hydrogen-carbon dioxide gas mixtures was removed by combustion. Samples of the cultures were taken after 40 and 64 hr incubation for estimation of growth. The results are recorded in Table 6. It may be seen that growth was scanty and delayed in the absence of oxygen; growth was also very slight with pure oxygen as the gas phase. Addition of carbon dioxide to the gas phase allowed more growth under anaerobic conditions. With a carbon dioxide tension in the gas phase varying in different jars from 38 to 53 mm, growth was then limited by the partial pressure of oxygen, being heaviest with a partial pressure of 720 mm.

(iii) *Methylene Blue and Ferricyanide as Final Electron Acceptors for the Oxidation of Glucose.*—As stated above, intact cell suspensions were incapable of fermenting glucose or glycerol. The ability of methylene blue or ferricyanide to replace oxygen as final electron acceptor was therefore tested in bicarbonate buffer with 5 per cent. carbon dioxide in nitrogen as the gas phase. With glucose as substrate, carbon dioxide was evolved at approximately the same rates in the presence of either acceptor. Methylene blue could not replace oxygen for the oxidation of glycerol. The oxidation of glucose with methylene blue as electron acceptor in place of oxygen was investigated in more detail. A Thunberg tube experiment, the results of which are set out in Table 7, showed that 4 moles of methylene blue were reduced per mole glucose oxidized. In manometric experiments with bicarbonate-carbon dioxide buffer at pH 7.4, a total of 8 moles of carbon dioxide were evolved per mole glucose oxidized. By measurements of the initial bicarbonate concentration, and the concentration remaining at the end of the reaction, it was shown that of the total of 8 moles of carbon dioxide evolved during the oxidation of 1 mole of glucose in bicarbonate, approximately 6 moles were due to acid formation, and 2 moles were due to the formation of carbon dioxide (Table 8). Clerici fluid was used as the manometer fluid in these experiments in order to measure the large gas evolution. In phosphate buffer, with nitrogen as the gas phase, 2 moles of carbon dioxide were evolved per mole glucose oxidized.

TABLE 7

METHYLENE BLUE AS ELECTRON ACCEPTOR IN GLUCOSE OXIDATION BY *A. MYCOIDES:* EFFECT OF METHYLENE BLUE CONCENTRATION

Thunberg tubes contained: bacterial suspension 0·5 ml; M/5 phosphare buffer 1·0 ml; methylene blue solution (stopper) 0·5 ml; M/100 glucose (stopper) 0·1 ml (1·0 μM)

Methylene Blue (μM)	Decolourization Time (min)
1·6	5
2·0	6
2·4	7·5
2·8	8·0
3·2	9·5
3·6	10
4·0	11·5
4·4	>30
4·8	>30

The results of these experiments are consistent with the equation for the oxidation of glucose:

$$C_6H_{12}O_6 + 4MB \longrightarrow 2CH_3COOH + 4MBH_2 + 2CO_2.$$

The reduced form of methylene blue, having a lower pK value than the oxidized form, displaces carbon dioxide from bicarbonate buffer at the pH of these experiments (pH 7.4), although not quantitatively.

DPN could also replace oxygen as electron acceptor for glucose oxidation. This was shown with frozen and thawed suspension in bicarbonate buffer with 5 per cent. carbon dioxide in nitrogen as the gas phase. In the presence of excess DPN a total of 4 moles of carbon dioxide were evolved per mole glucose oxidized.

(iv) *Catalase.*—Cell suspensions possessed catalase activity. Catalase activity was measured manometrically by oxygen evolution from hydrogen peroxide. A value, calculated from the rate over the first 5 min after tipping in substrate of Q_{O_2} (N) 800 was found at a temperature of 25°C.

TABLE 8

FORMATION OF ACID AND CARBON DIOXIDE IN GLUCOSE OXIDATION BY *A. MYCOIDES*, WITH METHYLENE BLUE AS ELECTRON ACCEPTOR

Warburg vessels contained: glucose 1 μM; methylene blue 5 μM; tested (*a*) in $NaHCO_3$ buffer with 5% CO_2 in N_2 (v/v) in gas phase (pH 7·4); acid estimated by bicarbonate disappearance; (*b*) in phosphate buffer (pH 7·4) with N_2 in gas phase

Values in μM

Experiment	Glucose Oxidized	HCO_3^-–CO_2 Buffer		Phosphate Buffer CO_2
		Acid	CO_2	
1	1·00	5·36	1·92	1·84
2	1·00	5·76	2·00	2·00

IV. DISCUSSION

The failure of earlier workers (Holmes and Pirie 1932; Warren 1942) to detect dehydrogenase activity in washed cells of the organism with glucose as substrate, and the low activities recorded for lactic dehydrogenase, may have been due either to excessive washing of the cells with consequent loss of coenzymes, to the growth conditions used, or to the age of the cells when harvested. In the present work the oxidative activity of the cells was comparatively high. Values for oxygen uptake, expressed as Q_{O_2} (N), of 300-400 were found regularly for the oxidation of all substrates attacked, with the exception of mannose.

Previous workers have investigated the formation of acid from carbohydrates etc. during growth by *A. mycoides*. Dujardin-Beaumetz (1900) found acid formation from glucose, fructose, and maltose. Nakamura, Futamura, and Watanuki (1926) added dextrin; Tang *et al.* (1935) mannose; and Turner, Campbell, and Dick (1935) starch. In the present work it was found that glucose, fructose, mannose, and glycerol were oxidized by cell suspensions, but not maltose, starch, or dextrin. It is possible that the oxidation of the latter substances requires adaptation.

The hexoses attacked (glucose fructose, and mannose), suggest that the initial step in their degradation is a phosphorylation by hexokinase.

Hexokinase is specific for the phosphorylation of these three sugars. Mannose is phosphorylated by yeast hexokinase at a considerably slower rate than are glucose and fructose. The rate of oxygen uptake found for mannose oxidation was considerably slower than that for glucose or fructose oxidation, suggesting that the rate of the initial phosphorylation reaction may limit the rate of mannose oxidation. The pathway for the oxidation of these hexoses, and the reasons for the absence of anaerobic glucose breakdown by intact cells are considered in a later paper (see page 37).

The oxidation of glycerol by a strain of *Streptococcus faecalis* which did not oxidize pyruvate has been investigated by Gunsalus and Umbreit (1945), who obtained evidence for the following mechanism for the oxidation by this organism. Glycerol was phosphorylated, probably to D-glycerophosphate. Glycerophosphate was oxidized by a flavoprotein enzyme reacting directly with oxygen to form D-glyceraldehyde phosphate and hydrogen peroxide. Further oxidation of triose phosphate was through the Embden-Meyerhof scheme to pyruvate, which in this organism was then reduced to lactate in order to regenerate oxidized diphosphopyridine nucleotide. In the absence of a mechanism for decomposing hydrogen peroxide (e.g. added catalase or pyruvate) the reaction ceased before all the glycerol was oxidized. With *A. mycoides* also the addition of catalase was necessary to allow the oxidation to continue. Furthermore, methylene blue could not replace oxygen for this oxidation. It is assumed that the pathway for glycerol oxidation is similar to that found by Gunsalus and Umbreit for *Strep. faecalis*, except that in *A. mycoides* pyruvate is further oxidized to acetate and carbon dioxide.

The overall equation demanded by this pathway:

$$C_3H_8O_3 + 1.5O_2 \longrightarrow CH_3COOH + CO_2 + 2H_2O,$$

agrees with the results obtained in the carbon balance experiment.

The nature of the terminal respiratory enzyme system catalysing the transfer of electrons from the reduced coenzymes to oxygen was not determined. Cytochrome absorption bands could not be detected, and the absence of oxidation of *p*-phenylene diamine—assuming the cell membrane to be permeable to this substance—indicates the absence of cytochrome *c* (Keilin and Hartree 1939). The formation of hydrogen peroxide during glucose, lactate, or pyruvate oxidation was not tested directly, but added catalase was without effect on these oxidations.

The almost complete absence of anaerobic growth may perhaps be explained by the absence of a mechanism for anaerobic glucose fermentation. The only energy-yielding reaction occurring anaerobically so far detected in the organism is the pyruvate dismutation reaction. It is suggested in a later paper that oxygen is required in glucose breakdown for the re-oxidation of reduced DPN. It might be possible to replace oxygen by other electron acceptors for growth purposes also.

The evidence for the absence of any mechanism for the oxidation or metabolism of the intermediate compounds of the Wood-Werkman scheme, or of the tricarboxylic acid cycle, appears conclusive. Acetate accumulated as the end-product of the oxidation of all substrates attacked, and none of the intermediate compounds of these systems was oxidized. That the method used of freezing and thawing the cells results in an alteration or destruction of permeability barriers was evident in experiments to be described later.

As a result of this general survey, it may be said that *A. mycoides,* although strongly aerobic in its growth requirements in the culture medium used, resembles in its metabolism the facultative organisms such as *Strep. faecalis* (strain 10C1) whose metabolism has been described by O'Kane (1950), rather than the strict aerobes. Although oxygen is required for growth, it is doubtful whether the organism derives any additional energy from the final electron transfer to oxygen.

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THE BREAKDOWN OF PYRUVATE BY *ASTEROCOCCUS MYCOIDES*, THE ORGANISM OF BOVINE PLEUROPNEUMONIA

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[*Manuscript received August* 12, 1953]

Summary

The pathway for pyruvate breakdown was investigated. Evidence was obtained that α-acetolactic acid does not participate in the pyruvate metabolism of this organism.

The pyruvate oxidation and dismutation systems were sensitive to inhibition by arsenite. Inorganic phosphate, Mg^{++}, or Mn^{++}, coenzyme *A*, diphosphopyridine nucleotide, cocarboxylase, and cysteine or glutathione were all required for maximum activity in the dismutation system. Although it was not possible to show an accumulation of acetyl phosphate it was concluded that pyruvate breakdown proceeds with the intermediate formation of acetyl coenzyme *A* and acetyl phosphate.

I. Introduction

It was shown previously that cell suspensions of *Asterococcus mycoides* oxidized pyruvate to acetate and carbon dioxide, and catalysed the anaerobic dismutation reaction of pyruvate to lactate, acetate, and carbon dioxide. Small amounts of acetoin were also formed anaerobically (Rodwell and Rodwell 1954). The mechanism for pyruvate breakdown is considered in this paper.

The pyruvate metabolism of many species of bacteria and of animal tissues has now been clarified. Korkes *et al.* (1950) and Korkes *et al.* (1951) analysed the pyruvate dismutation systems of *Escherichia coli* and *Streptococcus faecalis*. The complete system required cocarboxylase, Mg^{++} or Mn^{++}, coenzyme *A*, DPN, lactic dehydrogenase, pyruvic oxidase, and an acetyl acceptor system, e.g. oxalacetate and condensing enzyme, or inorganic phosphate and transacetylase. The part played by coenzyme *A* in acetyl transfer reactions has been the subject of recent reviews (Ochoa and Stern 1952; Welch and Nichol 1952).

Pyruvic oxidase of pigeon breast muscle was purified by Jaganathan and Schweet (1952). With ferricyanide as electron acceptor, the system required only cocarboxylase and Mg^{++} for pyruvate oxidation (Schweet and Cheslock 1952). The pyruvic oxidase of *Proteus vulgaris* also required only cocarboxylase, Mg^{++}, and a particulate cytochrome-containing fraction for the oxidation of pyruvate (Moyed and O'Kane 1952*a*, 1952*b*). Both preparations would acetylate and carry out the pyruvate dismutation reaction in the presence of coenzyme *A* and the necessary accessory enzymes, coenzymes, and acceptors.

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Krampitz (1951) suggested that α-acetolactic acid, which had been shown to be an intermediate in the formation of acetoin by *Staphylococcus aureus* (Watt and Krampitz 1947) and *Aerobacter aerogenes* (Juni 1950), might undergo a hydrolytic or phosphorylytic split to form acetic acid (or acetyl phosphate) and lactic acid, and thus become an intermediate in the dismutation reaction. Juni (1952) investigated this possibility, but could find no evidence for the participation of α-acetolactic acid in the dismutation reaction.

The mechanism suggested by Krampitz could be extended to include pyruvate oxidation. In the presence of lactic dehydrogenase, and an aerobic mechanism for the oxidation of reduced diphosphopyridine nucleotide, lactate, formed by the splitting of α-acetolactic acid, would be oxidized to pyruvate, which could then condense with another molecule of pyruvate and thus establish a cycle.

II. METHODS

The organism, growth conditions, and method of preparation of cell suspensions were the same as previously described (Rodwell and Rodwell 1954).

Dialysed enzyme extract.—The enzyme extract used to determine the cofactor requirements of the pyruvate dismutation system was prepared from lyophilized cells. One gram of lyophilized cells was ground in a chilled mortar for 5 min with 10 g 600-mesh alumina grinding powder ("Alloxite"), and 5.0 ml ice-cold phosphate buffer (pH 7.4). The mixture was extracted with 15 ml ice-cold phosphate buffer (pH 7.4) and centrifuged at 15,000 r.p.m. for 1 hr at 0°C. The clear yellow top layer was dialysed with constant agitation for 2 hr in cold water. Solid ammonium sulphate was added slowly to the dialysed extract to saturation. After centrifugation, the precipitate was dissolved in 5 ml water and the solution dialysed with agitation for 18 hr in cold water. The final extract contained 18 mg protein per ml (Biuret reaction using bovine albumin as standard).

α-Acetolactic acid.—This was prepared by the method of Krampitz (1948). The methyl ethyl ester was hydrolysed just before use with the equivalent amount of sodium hydroxide. α-Acetolactic acid remaining in manometer vessels at the end of the experiments was estimated manometrically by aniline citrate decarboxylation (Edson 1935).

Coenzyme A.—A concentrate of coenzyme *A* was prepared from pig liver by the method of Buyske *et al.* (1951) and its coenzyme *A* content assayed by the method of Handschumacher, Mueller, and Strong (1951).

Diphosphopyridine nucleotide (DPN).—A concentrate of DPN was prepared from yeast by the method of Williamson and Green (1940).

Cocarboxylase.—This was synthesized by the method of Weijlard and Tauber (1938).

Acetyl phosphate.—Acetyl phosphate was estimated by the method of Lipmann and Tuttle (1945).

III. EXPERIMENTAL

(*a*) *Metabolism of α-Acetolactic Acid*

Intact cell suspensions and cell suspensions which had been frozen and thawed were found to have no action on α-acetolactic acid at pH 7.4. The concentration of α-acetolactic acid remained constant during incubation, no carbon dioxide was evolved from the substrate anaerobically, nor was oxygen absorbed in its presence.

TABLE 1

EFFECT OF INHIBITORS ON PYRUVATE OXIDATION AND DISMUTATION REACTIONS

Intact cell suspensions. Rate of dismutation reaction determined in bicarbonate buffer with 5 per cent. CO_2 in N_2 (v/v) as gas phase (pH 7·4). Rate of oxidation reaction by oxygen uptake in phosphate buffer with air as gas phase. Rates calculated from linear portions of progress curves. Results expressed as percentage inhibition of rate in absence of inhibitor

Reaction	Inhibitor (M concentration)		Inhibition
Pyruvate dismutation	Arsenite	0·0001	39
	Arsenite	0·001	77
Pyruvate oxidation	Arsenite	0·00005	16
	Arsenite	0·0001	31
	Arsenite	0·00025	47
	Arsenite	0·0005	76
	Arsenite	0·001	86
Pyruvate oxidation	2,4-Dinitrophenol	0·0001	12
	2,4-Dinitrophenol	0·001	23
	2,4-Dinitrophenol	0·01	96
Pyruvate oxidation	Azide	0·0001	0
	Azide	0·001	20
	Azide	0·01	63
Pyruvate dismutation	Fluoride	0·04	6
	Fluoride	0·06	30
	Fluoride	0·08	57
	Fluoride	0·1	63
Pyruvate oxidation	Fluoride	0·005	95
	Fluoride	0·0075	100
Glucose oxidation	Fluoride	0·005	77
	Fluoride	0·0075	92
	Fluoride	0·01	100

(*b*) *Action of Inhibitors on Pyruvate Oxidation and Dismutation Reactions*

The effects of some inhibitors on the rates of the pyruvate oxidation and dismutation reactions with intact cell suspensions are shown in Table 1. Both reactions were sensitive to inhibition by arsenite. Pyruvate oxidation was relatively insensitive to inhibition by azide and by 2,4-dinitrophenol. The dismutation reaction was insensitive to fluoride. Pyruvate oxidation, in the experiment reported, was strongly inhibited by 0.005M fluoride. With the particular batch of suspension used in this experiment and in the absence of fluoride, there was a long lag period before oxygen uptake began, whereas the rate of the dismutation reaction was linear from the beginning. The same batch of suspension was used to determine the sensitivity of glucose oxidation to inhibition by fluoride. The lag period was shorter with glucose than with pyruvate. The results are included in Table 1 for comparison. Glucose oxidation was not as sensitive to fluoride inhibition as was pyruvate oxidation. With another batch of suspension which exhibited a shorter lag period, pyruvate oxidation was more resistant to fluoride; the rate was inhibited 76 per cent. by 0.02M fluoride and 92 per cent. by 0.04M fluoride. A possible reason for this variation in sensitivity is discussed later.

(*c*) *Formation of Acetyl Phosphate*

Several attempts were made to detect the formation of acetyl phosphate during pyruvate oxidation, and in the anaerobic dismutation reaction. Both intact cell suspensions and frozen and thawed suspensions were used. Fluoride, in concentrations ranging from 0.02 to 0.1M, was included as an inhibitor of phosphorylases. In none of the experiments was any acetyl phosphate detected.

(*d*) *Cofactor Requirements for the Dismutation Reaction*

The effect of various cofactor additions on the rate of the dismutation reaction was tested with the ammonium sulphate precipitated and dialysed enzyme extract. The results are set out in Table 2. It was not possible to measure a true rate in this experiment because of carbon dioxide retention. Bound carbon dioxide was liberated when the rate of the reaction appeared to be decreasing and the results are expressed as the amount of carbon dioxide evolved during a fixed interval (90 min). Nevertheless the figures may be accepted as an approximation to the rates. It may be seen that replacement of phosphate by veronal buffer, omission of Mg^{++} and Mn^{++}, or of the coenzyme *A* preparation all decreased the reaction appreciably. Omission of DPN and cocarboxylase as well as coenzyme *A* resulted in a further decrease. Omission of all the cofactor components and cysteine and glutathione reduced the amount of carbon dioxide to that obtained in the complete system in the absence of pyruvate.

IV. DISCUSSION

It is concluded that the pyruvate metabolism of *A. mycoides* proceeds with the intermediate formation of acetyl coenzyme *A*. Although the coenzyme preparations used were very impure, the presence of Mg^{++} or Mn^{++}, coenzyme *A* concentrate, DPN concentrate, cocarboxylase, and cysteine and glutathione were all required for maximum activity. Greater activity was found in phosphate buffer than in veronal buffer. It is considered unlikely that this effect was due to an inhibition of the reaction by veronal. In comparative experiments with undialysed extracts, no difference in the rate of the dismutation reaction was found with phosphate and veronal buffers.

No attempt was made to determine whether coenzyme *A* and DPN were required for pyruvate oxidation, but it is logical to consider that, in the intact cell at least, the only difference in the oxidation and dismutation systems lies in the manner in which reduced DPN is re-oxidized. The pyruvate metabolism of *A. mycoides* would then resemble that of *Strep. faecalis* as described by Korkes *et al.* (1951).

Table 2

COFACTOR REQUIREMENTS FOR PYRUVATE DISMUTATION SYSTEM

Complete system: enzyme extract (≡ 18 mg protein); phosphate (pH 7·4) 200μM; $MgCl_2$ 4μM; $MnCl_2$ 2·5μM; coenzyme *A* concentrate (Co*A*) 200μg; cysteine 20μM; glutathione (GSH) 10μM; pyruvate 20μM. Vol. 3·0 ml; gas phase, O_2-free N_2

Components	CO_2 (μl)
Complete system	181
Without—phosphate*	61
Without—Mg^{++}, Mn^{++}	156
Without—coenzyme *A*†	92
Without—Co*A*, DPN, cocarboxylase	80
Without—Mn^{++}, Mg^{++}, Co*A*, DPN, cocarboxylase, cysteine, GSH	13
Without—pyruvate	12

* Phosphate buffer replaced by veronal buffer (200μM).

† Coenzyme *A* concentrate replaced by DPN concentrate (= 0·04μM DPN) and cocarboxylase (approx. 0·1μM).

The sensitivity of both the oxidation and dismutation systems to inhibition by arsenite is consistent with a requirement for α-lipoic acid (Reed *et al.* 1951; Moyed and O'Kane 1952*b*).

It is considered unlikely that fluoride inhibits the oxidation system itself. Since the sensitivity was found to vary with the duration of the lag period, and since glucose oxidation was less sensitive than with pyruvate, it is probable that fluoride exerted its effect by inhibiting the biosynthesis of some components of the oxidizing system, e.g. a flavine nucleotide.

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THE PATHWAY FOR GLUCOSE OXIDATION BY *ASTEROCOCCUS MYCOIDES*, THE ORGANISM OF BOVINE PLEUROPNEUMONIA

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Summary

Cell suspensions of *A. mycoides* oxidize glucose but do not attack it anaerobically. The pathway for glucose oxidation was investigated.

Evidence was obtained for the presence of hexokinase and aldolase, and suspensions treated by freezing and thawing oxidized glucose-6-phosphate, frucose-6-phosphate, and fructose-1,6-diphosphate, but not gluconate or 6-phosphogluconate.

In contrast to intact cell suspensions, cell suspensions treated by freezing and thawing attacked glucose anaerobically at a slow rate. The rate was considerably increased when the reaction was linked with the reduction of acetaldehyde by yeast alcohol dehydrogenase. In the presence of arsenite, an inhibitor of the pyruvate dismutation reaction, intact cell suspensions catalysed a homolactic glucose fermentation anaerobically at a rate comparable with the rate of glucose oxidation.

It was concluded that glucose is broken down by *A. mycoides* by the Embden-Meyerhof pathway. Oxygen is required for the re-oxidation of reduced diphosphopyridine nucleotide.

I. INTRODUCTION

In the bacteria examined which oxidize glucose but are unable to attack it anaerobically, it has been found that glucose oxidation did not involve the Embden-Meyerhof scheme, but occurred either without phosphorylation (Barron and Friedemann 1941; Koepsall 1950; Stokes and Campbell 1951), or by a modification of the Warburg-Dickens hexose monophosphate pathway (Entner and Doudoroff 1952).

It was shown previously (Rodwell and Rodwell 1954*a*) that cell suspensions of a strain of *A. mycoides* oxidized glucose to acetate and carbon dioxide, but were incapable of attacking it anaerobically. Fructose and mannose were also oxidized, suggesting the participation of hexokinase in glucose degradation.

Pyruvate was oxidatively decarboxylated, and was attacked anaerobically by the dismutation reaction. Evidence was presented (Rodwell and Rodwell 1954*b*) that the dismutation system in *A. mycoides* is similar to that described in *Streptococcus faecalis*, i.e. a diphosphopyridine-linked, pyruvic-lactic dehydrogenase system requiring cocarboxylase, Mg^{++}, coenzyme *A*, and phosphate. Both the oxidation and the dismutation systems were strongly inhibited by arsenite.

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Glycerol, in the presence of added catalase, was oxidized to acetate and carbon dioxide, and was not attacked anaerobically. Methylene blue could not replace oxygen for glycerol oxidation. It was assumed that the pathway for glycerol oxidation resembled that described by Gunsalus and Umbreit (1945) for *Strep. faecalis*, i.e. glycerol is first phosphorylated to α-glycerophosphate which is oxidized by a flavoprotein enzyme to D-glyceraldehyde phosphate. Glyceraldehyde phosphate is then oxidized to pyruvate. In the organism studied by Gunsalus and Umbreit, pyruvate was reduced to lactate in order to regenerate DPN, whereas in *A. mycoides* it was assumed that pyruvate was further oxidized to acetate and carbon dioxide.

II. METHODS

The organism, growth conditions, and methods of preparation and standardization of cell suspensions, the preparation of frozen and thawed suspensions, and the manometric and chemical methods used have all been described previously (Rodwell and Rodwell 1954*a*). Cell-free extract was prepared by grinding lyophilized cells with "Alloxite" as described (Rodwell and Rodwell 1954*b*).

Hexokinase activity.—Hexokinase activity was tested manometrically, by carbon dioxide evolution from bicarbonate buffer with carbon dioxide in nitrogen as the gas phase, in the presence of glucose, ATP, and fluoride (Colowick and Kalcker 1943).

Alcohol dehydrogenase.—Crystalline alcohol dehydrogenase was prepared from yeast by the method of Racker (1950).

Glucose-6-phosphate.—Glucose-1-phosphate was prepared from starch by the action of potato phosphorylase (Hanes 1940) and then converted to glucose-6-phosphate with a crystalline preparation of phosphoglucomutase (Najjar 1948), following the procedure of Neifakl and Grechishkina (1951).

6-*Phosphogluconate.*—Glucose-6-phosphate was oxidized with bromine as described by Robison and King (1931).

Fructose-1,6-*diphosphate.*—A commercial sample of the tetrabasic barium salt was purified as the acid barium salt by the method of Neuberg, Lustig, and Rothenberg (1943).

Adenosine triphosphate (ATP).—Commercial ATP was purified by mercury precipitation.

All barium salts were converted to the sodium salts immediately before use in the experiments.

III. EXPERIMENTAL

(*a*) *Action of Inhibitors on Glucose Oxidation*

The effect of iodoacetic acid and sodium fluoride on the rate of glucose oxidation by cell suspensions in phosphate buffer is shown in Table 1. Glucose oxidation was sensitive to inhibition by both substances.

(*b*) *Hexokinase Activity*

Hexokinase activity was tested manometrically with suspension treated by freezing and thawing. The complete system contained fluoride (0.05M), Mg^{++} (0.01M), frozen and thawed suspension, glucose, ATP, bicarbonate, and 5 per cent. carbon dioxide in nitrogen (v/v) in the gas phase (pH 7.5). The rate of carbon dioxide evolution was constant only during the first 15 min after adding glucose and ATP. The rates, calculated from the first 15-min interval, are set out in Table 2.

Table 1

EFFECT OF INHIBITORS ON RATE OF GLUCOSE OXIDATION BY SUSPENSIONS OF *A. MYCOIDES*

Warburg vessels contained: M/5 phosphate buffer (pH 7·4) 1·25 ml; bacterial suspension 0·5 ml; inhibitor solution (or water) 0·25 ml (main compartment); M/20 glucose 0·25 ml (side bulb); 20% KOH (centre well)

Results expressed as percentage inhibition of rate of oxygen uptake

Inhibitor (M concn.)	Inhibition (%)
Iodoacetic acid 0·0001	29
Iodoacetic acid 0·001	82
Iodoacetic acid 0·01	100
Sodium fluoride 0·005	77
Sodium fluoride 0·0075	92
Sodium fluoride 0·01	100

Table 2

HEXOKINASE ACTIVITY OF *A. MYCOIDES*

Complete system: $NaHCO_3$ (0·078M) 1·0 ml; NaF (0·5M) 0·2 ml; $MgCl_2$ (0·1M) 0·2 ml; frozen and thawed bacterial suspension 0·6 ml; glucose (0·1M, side bulb) 0·2 ml; ATP (2 mg 7 min P/ml, side bulb) 0·4 ml; vol. 3·0 ml. Gas phase 7% CO_2 in N_2 (v/v). Rates expressed as μl CO_2/hr

Components	Rate
Complete system	396
Complete system without ATP	144
Complete system without glucose	168
Complete system without $MgCl_2$	166
Complete system with cysteine (M/300)	420

The results were consistent with the action of hexokinase. Carbon dioxide was evolved most rapidly in the complete system; Mg^{++} and cysteine both had a stimulatory effect. The results, however, were not conclusive because carbon dioxide was also evolved at a relatively rapid rate in the absence of either glucose or of ATP. The results were probably complicated by the presence of a fluoride-resistant ATP'ase, since acid-hydrolysable phosphorus (7 min P) had almost disappeared from the contents of all manometer vessels at the end of the experiment.

(*c*) *Aldolase Activity*

A suitable dilution of a cell-free extract was incubated in the test system of Sibley and Lehninger (1949), as set out in Table 3. The approximate activity determined by the colorimetric method, by the use of the standard curve of Sibley and Lehninger, agreed with the activity determined by alkali-labile phosphate determinations ($Q_{HDP} = 248$). The extract possessed relatively high aldolase activity compared with the figures published for various tissues by Sibley and Lehninger.

Table 3

ALDOLASE ACTIVITY OF *A. MYCOIDES*

Test system: Cell-free extract 1·0 ml (≡0·225 mg protein); 2-amino-2-methyl 1,3-propane diol—HCl buffer (0·1M, pH 8·6) 1·0 ml; hydrazine solution (0·56M, pH 8·6) 0·25 ml; fructose-1, 6-diphosphate (HDP) (0·45M, pH 8·6) 0·25 ml. Incubation period: 30 min at 37° C. Reaction stopped and mixtures deproteinized by addition of 2 ml 10% (w/v) trichloracetic acid solution (TCA). Samples of TCA supernatants taken (*a*) for colorimetric method, 0·1, 0·2, 0·3 ml; (*b*) for alkali-labile P determinations, 0·5 ml

	Colorimetric Method	Alkali-labile P
HDP split/mg protein/hr (μl)	258	248

(*d*) *Oxidation of Phosphorylated Hexose Intermediates*

The oxidation of phosphorylated hexose intermediates by intact cell suspensions was tested in phosphate buffer. Oxygen uptake was rapid with glucose, but with glucose-6-phosphate, fructose-6-phosphate, hexosediphosphate, gluconate, or 6-phosphogluconate it was no higher than the endogenous rate.

The oxidation of the phosphorylated hexoses by suspensions treated by freezing and thawing was tested in bicarbonate buffer with 5 per cent. (v/v) carbon dioxide in nitrogen as the gas phase, and excess methylene blue in place of oxygen as electron acceptor. This was done because suspensions treated by freezing and thawing often had impaired ability to take up oxygen, but retained their ability to oxidize glucose with methylene blue as electron acceptor. With methylene blue as electron acceptor, the oxidation of 1 mole of glucose results in the evolution of 8 moles of carbon dioxide from bicarbonate buffer (Rodwell and Rodwell 1954*a*). Carbon dioxide evolution from glucose, glucose-6-phosphate, fructose-1,6-diphosphate, and 6-phosphogluconate is shown graphically in Figure 1.

Glucose, hexosediphosphate, and glucose-6-phosphate were attacked at comparable rates and the amount of carbon dioxide evolved approached the theoretical value, whereas the rate of carbon dioxide evolution from 6-phosphogluconate was no faster than in the absence of substrate. In other experiments fructose-6-phosphate was also shown to be attacked by frozen and thawed suspensions.

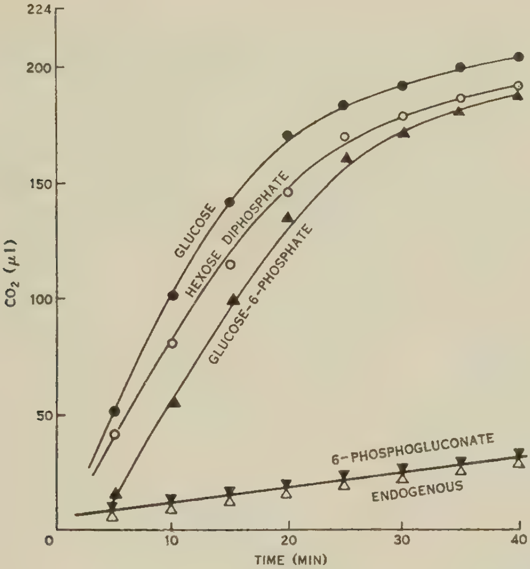

Fig. 1.—Oxidation of phosphorylated hexose intermediates by frozen and thawed suspension of *A. mycoides*. Manometer vessels contained: frozen and thawed bacterial suspension 1.5 ml; $NaHCO_3$ 50 μM; substrate 1.25 μM; coenzyme concentrate (fraction *A*) 1 mg; methylene blue 5 μM. Volume of fluid 2.5 ml; gas phase 7% CO_2 in N_2 (v/v).

(*e*) *Anaerobic Glucose Breakdown by Frozen and Thawed Suspensions*

In contrast to intact cell suspensions, suspensions treated by freezing and thawing were capable of glucose breakdown anaerobically. The rate was slow in comparison with the rate of glucose oxidation. The rate of anaerobic hexose breakdown by suspensions treated by freezing and thawing may be compared with the rate for the oxidation of the substrates with methylene blue as electron acceptor, in Table 4 (for hexosediphosphate breakdown) and in Table 5 (for glucose breakdown).

(*f*) *Effect of the Addition of Pyruvate on the Rate of Anaerobic Hexose Breakdown*

The effect of the addition of pyruvate on the rate of anaerobic hexose degradation by suspension treated by freezing and thawing was tested by the rate of carbon dioxide evolution from bicarbonate buffer. Hexosediphosphate was used as hexose substrate in this experiment. The results are shown in Table 4. It may be seen that the rate of carbon dioxide evolution when both substrates were present (156 μl/hr) was slightly greater than the sum of the rates for the pyruvate dismutation reaction and for hexosediphosphate breakdown in the absence of added pyruvate (120 μl/hr).

Table 4

EFFECT OF THE ADDITION OF PYRUVATE ON THE RATE OF ANAEROBIC BREAKDOWN OF HEXOSEDIPHOSPHATE BY FROZEN AND THAWED SUSPENSIONS OF *A. MYCOIDES*

Warburg vessels contained: Main compartment: M/10 $NaHCO_3$ 0·4 ml; frozen and thawed suspension 0·5 ml; M/30 iodoacetic acid (IAA) 0·25 ml; side bulb: M/20 hexosediphosphate (HDP) 0·2 ml; M/50 methylene blue (MB) 0·3 ml; M/50 pyruvate 0·2 ml; M/10 $NaHCO_3$ 0·1 ml. Volume fluid 2·5 ml. 5% CO_2 in N_2 in gas phase

Components	Rate (μl CO_2/hr)
No substrate	20
Pyruvate	68
HDP	52
Pyruvate+HDP	156
Pyruvate+HDP+IAA	20
HDP+MB	286

(*g*) *Effect of Linking the Reduction of Acetaldehyde by Yeast Alcohol Dehydrogenase with Anaerobic Glucose Breakdown*

The complete system contained yeast alcohol dehydrogenase, frozen and thawed suspension, acetaldehyde, and bicarbonate buffer with 5 per cent. (v/v) carbon dioxide in nitrogen as the gas phase. The rates of carbon dioxide evolution obtained in two experiments are set out in Table 5. It may be seen that, in the presence of both alcohol dehydrogenase and acetaldehyde the rate of glucose breakdown was increased considerably in both experiments. The addition of alcohol dehydrogenase alone increased the rate of glucose breakdown, slightly in one experiment, and considerably in the other experiment. The addition of acetaldehyde alone depressed the rate slightly.

(*h*) *Effect of Arsenite on Anaerobic Glucose Breakdown*

Both the oxidation and the anaerobic dismutation of pyruvate by intact cell suspensions were strongly inhibited by arsenite (Rodwell and Rodwell 1954*b*). In the presence of arsenite it was found that intact cell suspensions catalysed a homolactic glucose fermentation anaerobically. The effect of the concentration of arsenite on the rate of the reaction in bicarbonate buffer is shown in Figure 2. The rate of oxygen uptake in phosphate buffer is included in Figure 2 for comparison. It may be seen that in the presence of a concentration of

0.03M arsenite the rate of carbon dioxide formation approached the rate of oxygen uptake. That the fermentation in the presence of arsenite was homolactic was confirmed in another experiment in which lactic acid was estimated colorimetrically in the contents of the manometer vessels. Of the carbon dioxide evolved from bicarbonate buffer in the presence of 0.03M arsenite, 96 per cent. was accounted for as lactic acid.

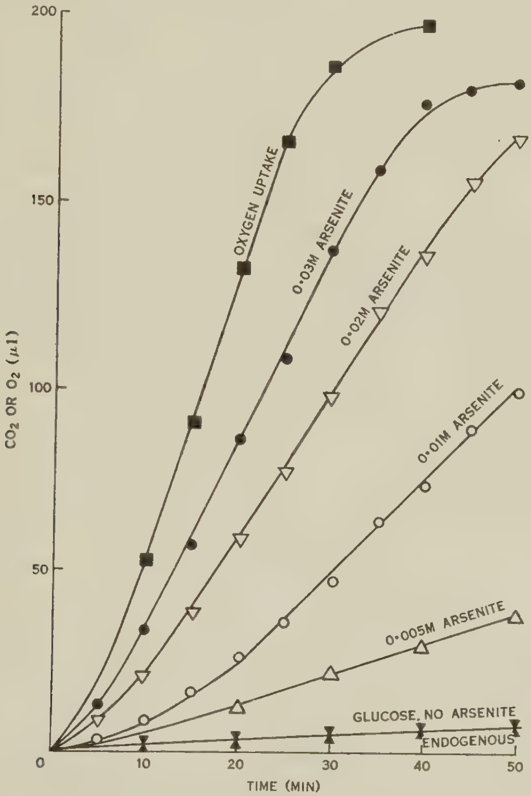

Fig. 2.—Effect of arsenite on rate of anaerobic glucose breakdown by intact cell suspensions of *A. mycoides*. *Anaerobic breakdown*: 0.075M $NaHCO_3$ 0.5 ml; bacterial suspension 0.5 ml; arsenite solution 0.25 ml; M/20 glucose 0.1 ml; water to 2.5 ml. 5% CO_2 in N_2 in gas phase. *Oxygen uptake*: M/5 phosphate buffer (pH 7.4) 1.5 ml; bacterial suspension 0.5 ml; M/20 glucose 0.1 ml; 20% KOH 0.2 ml (centre well).

IV. Discussion

The sensitivity of glucose oxidation to inhibition by iodoacetic acid implies the participation of a DPN or TPN dehydrogenase in glucose breakdown. Fluoride may not have exerted its effect by inhibiting enolase activity, but may have inhibited the biosynthesis of a factor required by the terminal respiratory system as was suggested for the inhibition of pyruvate oxidation (Rodwell and Rodwell 1954*b*).

Although the presence of hexokinase was not demonstrated conclusively, the range of hexoses attacked, the oxidation of the phosphorylated hexose intermediates of the Embden-Meyerhof-Parnas scheme, and the presence of aldolase provide further evidence. It was concluded that the initial step in the breakdown of glucose, fructose, and mannose was a phosphorylation to glucose-6-phosphate, fructose-6-phosphate, and mannose-6-phosphate respectively, and that mannose-6-phosphate would be converted to fructose-6-phosphate by the action of phosphohexoisomerase. Since fructose-1,6-diphosphate was attacked, and the organism was shown to possess aldolase activity, and since gluconate or 6-phosphogluconate were not attacked, it was concluded that the breakdown of hexoses proceeds through fructose-1,6-diphosphate.

Table 5

EFFECT OF LINKING ANAEROBIC GLUCOSE BREAKDOWN BY FROZEN AND THAWED SUSPENSION OF *A. MYCOIDES* WITH REDUCTION OF ACETALDEHYDE BY YEAST ALCOHOL DEHYDROGENASE

Complete system: Frozen and thawed suspension ≡ approx. 1·5 mg N; $MgCl_2$, 30μM; alcohol dehydrogenase (Al D) solution in M/20 phosphate buffer containing 0·1% bovine albumin 0·4 ml; glucose 5μM; acetaldehyde 10μM; $NaHCO_3$ 60μM; fluid vol. 3·0 ml; 7% CO_2 in N_2 (v/v) as gas phase

Components	Rate (μl CO_2/hr)	
	Expt. 1	Expt. 2
Complete system	135	233
Complete system without Al D*	59	61
Complete system without CH_3CHO	80	120
Complete system without Al D, CH_3CHO	70	63
Complete system without glucose	13	28
Complete system without glucose, Al D*, CH_3CHO	8	26
Complete system without Al D, CH_3CHO; with methylene blue (6 μM)	280	

* Alcohol dehydrogenase solution replaced by M/20 phosphate buffer containing 0·1% bovine albumin.

The pathway for the oxidation of triose phosphate must now be considered. If glyceraldehyde phosphate is oxidized through the Embden-Meyerhof scheme, i.e. by a triose phosphate dehydrogenase specific for DPN, an explanation must be found for the absence of anaerobic glucose breakdown by intact cell suspensions. It was concluded previously that the pyruvate dismutation system of *A. mycoides* resembled the system described in other organisms. In the dismutation system, $DPNH_2$ formed by the oxidation of pyruvate is re-oxidized by the reduction of pyruvate by lactic dehydrogenase. It was difficult to see why the reduction of pyruvate by lactic dehydrogenase could not serve to re-oxidize $DPNH_2$ formed by the oxidation of glyceraldehyde phosphate in the glycolysis system also. Another possible explanation for the absence of anaerobic glucose breakdown might be that triose phosphate is oxidized by an enzyme requiring an electron carrier other than DPN, e.g. TPN (Tewfik and Stumpf 1951; Gibbs 1952; Arnon 1952), and that oxygen or other electron acceptor was required for the re-oxidation of $TPNH_2$. This would not explain why suspensions treated by freezing and thawing were capable of a slow anaerobic glucose breakdown.

No attempt was made to isolate a triose phosphate dehydrogenase from *A. mycoides,* and to determine its coenzyme specificity, but indirect evidence was obtained that triose phosphate is oxidized by a DPN-specific enzyme. When anaerobic glucose breakdown by suspensions treated by freezing and thawing was linked with an external system for the oxidation of $DPNH_2$, viz. the reduction of acetaldehyde by purified yeast alcohol dehydrogenase, the rate of anaerobic glucose breakdown was very much increased. This experiment suggests that the rate of anaerobic glucose breakdown by frozen and thawed suspension is limited by the rate at which $DPNH_2$ is re-oxidized. It might be expected that in this system, pyruvate would undergo dismutation to lactate, acetate, and carbon dioxide. The overall equation for the reaction would then be:

$$C_6H_{12}O_6 + 2CH_3CHO + H_2O = CH_3COOH + CH_3CHOH.COOH + CO_2 + 2CH_3CH_2OH.$$

It would therefore be expected that for each mole of glucose broken down, 3 moles of carbon dioxide would be evolved from bicarbonate buffer, and 1 mole of carbon dioxide would be formed in phosphate buffer. The amounts of carbon dioxide found varied in different experiments and did not reach the theoretical value demanded by the equation. However, more than 2 moles in bicarbonate buffer and approximately 0.6 moles in phosphate buffer were recorded. The incomplete yield of carbon dioxide may have been due to aldehyde toxicity. Acetaldehyde, in the absence of alcohol dehydrogenase, slightly depressed the rate of the reaction.

An analogy may be drawn between glucose fermentation in *A. mycoides* and Neuberg's forms of glycerol fermentation in yeast. In the third form of glycerol fermentation, in which the fermentation is carried out at an alkaline pH, acetaldehyde is removed by a dismutation reaction, $DPNH_2$ is then reoxidized by the reduction of dihydroxyacetone phosphate by α-glycerophosphate dehydrogenase. It is postulated that in *A. mycoides,* pyruvate is removed by the pyruvate dismutation reaction, but the organism possesses no other anaerobic mechanism for the reoxidation of $DPNH_2$, and fermentation cannot occur. The glycolytic and pyruvate dismutation systems compete for pyruvate, lactic dehydrogenase, and DPN. In the presence of added pyruvate, or of another system for the oxidation of $DPNH_2$, the rate of anaerobic hexose breakdown by frozen and thawed suspensions was increased. In the presence of arsenite, a potent inhibitor of the pyruvate dismutation reaction, intact cell suspensions catalysed a homolactic glucose fermentation at a rate comparable with the rate of glucose oxidation.

A number of explanations are possible for the difference between organisms such as *Strep. faecalis,* which possess the pyruvate dismutation system and yet catalyse a homolactic glucose fermentation, and *A. mycoides*. There might be differences between the relative concentrations of pyruvic oxidase and lactic dehydrogenase, differences in the relative affinities of the enzymes for pyruvate, or differences in the organization within the cells of the two species. The latter possibility appears plausible. Treatment of suspensions by freezing and thawing resulted in a lowering of the rate of the pyruvate dismutation reaction, and suspensions so treated were capable of a slow anaerobic glucose breakdown. The freezing and thawing treatment may have resulted in a disturbance of the intracellular organization, which allowed pyruvate to combine with lactic dehydrogenase rather than pyruvic oxidase.

V. Acknowledgment

We wish to thank Dr. A. W. Turner, Assistant Chief of Division, for his interest and encouragement.

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RUGOSE LEAF CURL — A NEW VIRUS DISEASE TRANSOVARIALLY TRANSMITTED BY THE LEAFHOPPER *AUSTROAGALLIA TORRIDA*

By N. E. Grylls *

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Summary

A plant virus disease, not previously described, was obtained from the leafhopper *Austroagallia torrida* Evans collected from lucerne fields in Canberra. Symptoms have not been recognized in the field, but 16 species of plants from eight families developed symptoms in the glass-house.

The most characteristic symptoms included severe rugosity, twisting and curling of leaves, and marginal chlorosis. In addition, vein-clearing or netting, interveinal chlorosis, proliferation of shoots, and the production of stunted flowers were observed in various hosts. Anatomical studies of the stems of infected tomato and *Physalis floridana* Rydb. have indicated that the virus affects the phloem. The disease was not transmitted by mechanical means nor by *Cuscuta* but it was transmitted by grafting and by *A. torrida*. The virus was transmitted transovarially by this leafhopper.

I. INTRODUCTION

During a search for vectors of lucerne witches' broom virus more efficient than *Orosius argentatus* (Evans), a number of species of leafhopper were brought to the laboratory from lucerne fields. Attempts were made to establish virus-free colonies of these insects by placing nymphs as they hatched on virus-free seedlings, according to the method of Stahl and Carsner (1916).

One of the species investigated was *Austroagallia torrida* Evans. This species had been found in considerable numbers in fields containing lucerne witches' broom, and mild virus symptoms were observed on *Datura tatula* L. during attempts to establish a colony of the leafhopper on this plant. Collections of *A. torrida* were therefore made from a lucerne field at the C.S.I.R.O. Experiment Station, Dickson, A.C.T., from January to April 1951. Disease symptoms appeared on a number of plants on which these insects were fed. The symptoms were distinct from those of lucerne witches' broom, and indeed from any virus previously described. Later, the same disease was obtained from *A. torrida* collected from Hawkesbury Agricultural College, Richmond, N.S.W. Leafhoppers collected in April 1952 did not transmit symptoms of the virus to susceptible food plants on which they were fed for several months; but in March 1953, two carrot seedlings out of six, on each of which groups of 20 adults from the next generation were caged, developed typical symptoms of the disease. It seemed possible, therefore, that a new virus had been obtained and that it had

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been transmitted through the egg of the leafhopper. This paper describes experiments demonstrating that this suggestion is correct. The name rugose leaf curl virus is proposed for the pathogen. It has affinities with viruses grouped by Holmes (1948) in the genus *Aureogenus* Black.

II. HOST RANGE OF THE DISEASE

The following plants have been found to show distinct symptoms when infected with the virus of rugose leaf curl by insect transmission:

Solanaceae: *Lycopersicon esculentum* (Mill.), tomato, var. Rouge de Marmande and Kondine Red.
    Datura stramonium L.
    D. tatula L.
    Physalis floridana Rydb.
    Nicandra physaloides L. Gaertn.
Malvaceae: *Malva parviflora* L.
Umbelliferae: *Daucus carota* L., carrot, var. Chantenay.
    Peucedanum sativum (L.) Benth. & Hook., parsnip, var. Hollow Crown.
Leguminosae: *Medicago sativum* L.
    M. lupulina L.
    Trifolium pratense L.
    Crotalaria sp.
Compositae: *Sonchus oleraceus* L.
Urticaceae: *Urtica urens* L.
Polygonaceae: *Polygonum aviculare* L.
Rosaceae: *Sanguisorba minor* Scop.

Species which did not show symptoms when transmission by graft or by *A. torrida* was attempted were:

Solanaceae: *Nicotiana tabacum* L.
    N. glutinosa L.
    Solanum melongena L.
    S. tuberosum L.
    Capsicum annuum L.
Compositae: *Callistephus chinensis* Nees.
Chenopodiaceae: *Beta vulgaris* L., var. silver beet.
    B. vulgaris L., var. sugar beet.*
Apocynaceae: *Vinca alba* L.

III. SYMPTOMATOLOGY

The symptoms of rugose leaf curl on a number of hosts are illustrated in Plates 1-4. In most hosts the symptoms are severe and unmistakable, being characterized by extreme rugosity of affected leaves and marked leaf curling. The period before symptoms appeared was shortest during the hottest and brightest months. The shortest time between infection and the appearance of symptoms at any period was 32 days in tomato and the longest period was 123 days on red clover.

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* Seven out of 12 plants fed on by *A. torrida* in groups of 10 per indicator developed mild proliferation. Five to seven months later crown necrosis developed which destroyed the main growing tip. The remaining shoots remained small and stunted but no other leaf symptoms were evident. These were not considered definite symptoms of rugose leaf curl.

(*a*) *Solanaceae*

(i) *Tomato*.—The symptoms on both varieties of tomato tested were the same. They consisted of marginal and interveinal chlorosis, followed by a distinct vein-clearing resulting in a netted appearance. Often these two symptoms appeared simultaneously. The leaves then curled downward from the margins and became puckered or rugose. Flowering sometimes occurred before the appearance of symptoms. However, when normal flowers were not produced before the symptoms appeared, flowering was delayed and abnormal. In some instances green buds formed with the pistil protruding up to 3/16 in. from the tip (Plate 1, Fig. 1). These buds did not usually produce flowers and soon fell off. One plant which produced these buds formed flowers 12 months after the appearance of symptoms. These were small but normal in colour and shape, and fruits formed, ripened, and produced viable seed. No symptoms appeared on plants grown from this seed, suggesting that the virus is not transmitted through the seed. In all instances where flowers formed on infected plants they were smaller than normal. Proliferation of shoots occurred from the leaf axils, resulting in a bushy appearance. Plate 1 illustrates the symptoms on tomato. A study of the anatomy of an infected tomato stem showed proliferation of the phloem tissue and hypertrophy of cells. Similar symptoms were noted in histological studies of tomato big bud virus (Samuel, Bald, and Eardley 1933). No root symptoms were observed.

(ii) Datura *spp*.—The symptoms were similar on both *D. stramonium* and *D. tatula*. Again the first symptoms were marginal and interveinal chlorosis. Vein-clearing was not as distinct as in tomato but rugosity was very pronounced (Plate 3, Fig. 3). The twisting and curling downwards from the leaf margins were very conspicuous and plants developed the characteristic puckered or rugose appearance.

(iii) Nicandra physaloides.—Symptoms on this host were also characterized by marginal and interveinal chlorosis and distinct vein-clearing. Rugosity was very pronounced and proliferation occurred from the leaf axils. The new shoots showed definite symptoms of the virus.

(iv) Physalis floridana.—Early symptoms on this host were similar to those on tomato. Marginal and interveinal chlorosis was followed by distinct vein-clearing. Twisting and curling of leaves occurred and infected plants became stunted and died. Severe hypertrophy of the phloem and collapse of phloem cells were observed in histological preparations (Plate 4, compare Figs. 2 and 3). The observations on this host and on tomato suggest that rugose leaf curl is a phloem-inhabiting virus. An attempt was made to study phloem necrosis on this host and tomato by the coriphosphine and rhodamine B test (Keleny 1951), but no necrosis like that seen in plants infected with potato leaf roll virus was observed by ultraviolet illumination.

(*b*) *Malvaceae*

Malva parviflora showed the characteristic rugose leaf curl symptoms. Marginal and interveinal chlorosis was closely followed by distinct vein-clearing and curling from the margins and veins. Plants infected at an early stage remained stunted and all new growth exhibited a rugose appearance. Symptoms often appeared on almost fully expanded leaves before they were evident on the growing tip (Plate 3, Fig. 4). Small flowers were formed. These set viable seed, but again no symptoms have been noted on plants grown from this seed. No symptoms were noted on the roots.

(*c*) *Umbelliferae*

The virus was transmitted to two species of plants in this family. Carrot (var. Chantenay) was a good indicator plant for rugose leaf curl. Carrot plants growing vigorously before infection showed a marked reaction. First symptoms generally appeared on the growing tip, producing a fern-frond appearance (Plate 2, Fig. 1). Leaflets then developed vein-clearing, twisted, and curled, and the plants became very stunted. Flowering was advanced, and abnormal umbels were formed at from 1 to 4 in. from the crown. Slight proliferation occurred in some cases but the characteristic symptoms were the twisting and curling and the stunting of plants (Plate 2, Fig. 2). Woolly secondary roots were formed and the plants developed a soft rot and died within 3 months of infection.

Parsnip: Symptoms on this host were very similar to those on carrot but the plant died before flowering.

(*d*) *Leguminosae*

On legumes the most outstanding symptom was marginal chlorosis and rugosity, although red clover exhibited severe twisting and curling of leaves and marked stunting (Plate 3, Fig. 1). On lucerne, symptoms were sometimes indistinguishable from those produced by a strain of lucerne witches' broom described by Helms (unpublished data). Early symptoms on lucerne were marginal chlorosis, rounding and puckering of leaves, and shortening of the internodes (Plate 4, Fig. 1). Infected plants produced thin shoots and remained dwarfed. Similar symptoms also appeared on *Crotalaria* sp.

(*e*) *Compositae*

Symptoms on *Sonchus oleraceus* consisted of marked vein-clearing, marginal and interveinal chlorosis, followed by rugosity and curling of leaves (Plate 3, Fig. 2).

(*f*) *Urticaceae*

Urtica urens showed symptoms of marginal and interveinal chlorosis and leaf curl. Symptoms were not distinctive owing to the light yellowish green colour of this host when grown under glass-house conditions.

(*g*) *Polygonaceae*

Polygonum aviculare exhibited marginal chlorosis and characteristic twisting and curling of the leaves.

(*h*) *Rosaceae*

Sanguisorba minor infected by rugose leaf curl virus developed vein-clearing and rugosity.

IV. EXPERIMENTAL TRANSMISSION OF THE DISEASE

(*a*) *Attempts at Mechanical Transmission*

Inoculation of finely ground leaves of tomato infected with the rugose leaf curl virus in buffer solution to healthy tomatoes, using the usual carborundum method, proved unsuccessful. Therefore, a larger experiment was set up using the methods outlined by Hougas (1951), which provided optimal conditions for infection with potato yellow dwarf virus.

Eighty young tomato seedlings were submitted to a pre-inoculation darkening period of approximately 24 hr. One gram of infected tomato plant, including leaves and the growing tip, which had recently developed typical symptoms of the disease after infection by viruliferous *A. torrida*, was finely ground in buffer solution to which had been added cysteine hydrochloride to give a concentration of 0.01M in the inoculum. Forty plants were inoculated by the carborundum method and 40 by the pin-puncture technique. Twenty punctures were made in the main stalk near the growing tip of each plant in the latter group, the pin being dipped in the inoculum between each insertion. The same inoculum was used for each test and groups of 20 plants of each series were inoculated alternately. The plants were held for about 5 months, together with 20 control plants under conditions comparable with those under which transmission was obtained by the leafhopper vectors within the same period. No symptoms developed on any of the test plants.

(*b*) *Attempts at Transmission by Dodder*

Cuscuta campestris Yunkers was established on infected red clover and tomato plants. No transmission of virus occurred when dodder from these plants was transferred to or connected to various host plants. Plants tested were tomato, carrot, red clover, and *Malva*. No symptoms of the virus were evident on any of these plants during a period of 6 months, although dodder was growing vigorously on all of them.

(*c*) *Graft Transmission*

The rugose leaf curl virus was successfully transmitted by the irrigated scion graft technique to tomato and *Datura*. Attempts to transmit it by graft from tomato to *N. tabacum* and *N. glutinosa* were unsuccessful although the grafts made satisfactory union and remained alive for approximately 3 wk. With potato grafts, successful union was made and the scions remained vigorous for several months but no symptoms were observed. The grafts listed in Table 1 were all considered to have made union sufficient to give transmission of the virus. Transmission occurred from *Datura* to tomato and from tomato to tomato although the scions died after 14 days. Transmission was accomplished by grafting a tomato scion from a graft-infected plant. Thus it appears certain that the symptoms were not due to a toxin of insect origin.

TABLE 1

GRAFT TRANSMISSION OF THE RUGOSE LEAF CURL VIRUS

Source of Virus	Plant Grafted	Number of Grafts	Number of Transmissions	Mean Time for Appearance of Symptoms
Tomato	Tomato	12	8	51 Days (limits 34-96 days)
Tomato	*N. tabacum*	6	0	—
Tomato	*N. glutinosa*	6	0	—
Tomato	Potato var. 11-84	3*	0	
	Potato var. Sebago	3	0	—
	Potato var. Katahdin	3	0	
Tomato	*Datura* spp.	3	1	152 Days
D. tatula	*D. tatula*	2	1	56 Days
D. tatula	Tomato	3	2	105 Days (limits 63-147 days)

*Spindly shoots have grown out of tubers of two of these plants. No leaf symptoms recognizable as rugose leaf curl were evident.

(i) *Attempts to Transmit by* O. argentatus (*Evans*).—*O. argentatus*, shown by Helson (1950) to be a vector of lucerne witches' broom virus, was tested for its ability to transmit the new disease to carrot, tomato, and *Malva*. Groups of virus-free *O. argentatus* were caged on tomato, red clover, and parsnip plants showing distinct symptoms of the virus disease, for a period of 7 days. They were then caged in groups of 10 per plant on three different species of indicator plants, tomato, carrot, and *Malva*, for a further 7 days. Twelve carrot, eight tomato, and eight *Malva* indicator plants were used but no virus symptoms occurred. The indicator plants were retained for approximately 5 months, the tests being carried out at a time when transmission was obtained to similar indicators with *A. torrida* as the vector.

(ii) *Details of Transmission by* A. torrida.—Several attempts were made to determine the ability of *A. torrida* to transmit the virus of lucerne witches' broom, but in each experiment the symptoms that developed were those of rugose leaf curl and were identical with those obtained from *A. torrida* that had not had access to the virus of lucerne witches' broom. There is no evidence that the virus of rugose leaf curl was obtained from this lucerne. It is more likely that it was present in the leafhopper stock.

In experiment 1, virus was transmitted with inoculation feeding periods of from 24 hr to 4 days. The percentage of plants infected and the time taken for symptoms of rugose leaf curl to appear are given in Table 2.

In experiment 2, groups of 10 leafhoppers were used, with feeding periods of from 15 min to 4 days, on rugose leaf curl plants, with similar periods on indicator plants.

TABLE 2

INDICATOR PLANTS INFECTED WITH RUGOSE LEAF CURL BY *A. TORRIDA* FOLLOWING FEEDING UP TO 4 DAYS ON LUCERNE WITCHES' BROOM

Indicator Species	Number of Plants	Number Infected	Percentage Infection	Time to First Appearance of Symptoms (days)
Carrot	35	25	71·4	45–84
Tomato	25	13	52	32–110
D. tatula	16	8	50	60–80
Red clover	50	3	6	88–223

This demonstrated that infections did not occur unless the insects fed on the indicator plants for longer than 30 min (Table 3). This is probably the time required for the insects to reach the phloem tissue.

TABLE 3

NUMBER OF TOMATO PLANTS INFECTED WITH RUGOSE LEAF CURL AFTER VARYING PERIODS OF FEEDING BY *A. TORRIDA*

Duration of Inoculation Feeding	15 Min	30 Min	60 Min	19 Hr	48 Hr
Number of plants infected out of 12	0	0	2	9	7

V. TRANSOVARIAL INFECTION

The most interesting feature of transmission of rugose leaf curl virus by *A. torrida* is the fact that it was transmitted through the egg of this vector. This phenomenon was demonstrated in two series of experiments. The experience of the first season indicated that this occurred but in view of the importance of establishing it beyond doubt, detailed experiments were undertaken in the 1952-53 season. These experiments confirmed that the virus was transmitted by the leafhopper to its progeny.

Test 1.—Infections by single newly hatched nymphs. Twenty-five *A. torrida* adults were fed for 4 days on a red clover plant infected with rugose leaf curl.

The 13 surviving adults were then transferred to a *M. parviflora* plant to oviposit, and 1 month later nymphs began to emerge from the stems and petioles of this plant. Emergence generally took place in the morning, commencing about an hour or so after sunrise. Care was taken to ensure that the emerging nymphs had not fed before they were transferred to healthy plants. After 35 days 60 nymphs had been transferred singly to single indicator plants. These nymphs became adult in approx. 30 days and were then removed from the indicator plants. After approx. 3 months, 25 of the 60 indicator plants, or 42 per cent., showed symptoms of the disease.

Test 2.—Twenty-five adults were added to the surviving insects of the above breeding colony after a similar acquisition feeding period to those in Test 1. At certain periods there were more newly hatched nymphs available than indicator plants and they were therefore placed on another 50 *Malva* plants in groups of five. Symptoms appeared on 37, or 74 per cent., of the indicators. The mean time for symptoms to appear was 79 days, with limits of 57 to 102 days. The mean time for symptoms to appear was shorter and the percentage of plants infected was higher than in the previous test, probably owing to the fact that conditions for transmission improved later in the season, and perhaps also to a dosage effect. Symptoms appeared on some of these plants before the leafhoppers were removed but others appeared after the insects were removed.

VI. THE VECTOR AUSTROAGALLIA TORRIDA

(*a*) *Taxonomy*

A. torrida was described by Evans (1935) from specimens collected at Adelaide, S.A. Evans (1940) transferred the species to the genus *Nehela* (Buchanan White 1878). More recently, on further examination of the species, in conjunction with Dr. China of the British Museum, he now considers it should revert to *Austroagallia torrida* Evans (personal communication).

(*b*) *Distribution*

It seems likely that *A. torrida* occurs in every mainland State of Australia. Specimens have been identified from Queensland; Richmond and several localities in the Lachlan Valley, N.S.W.; Canberra, A.C.T.; Katherine, Northern Territory; Adelaide, S.A.; and the Ord River area, Wiluna, and Nedlands, W.A. The species is the only known member of the subfamily in Australia and Evans (personal communication) considers that it was introduced into Australia, probably from the Mediterranean region.

(*c*) *Life History*

The food plants most favoured in the laboratory include *Malva*, *Datura*, red clover, lucerne, and carrot, on all of which it feeds and breeds freely. Under insectary conditions there appear to be three complete but overlapping generations. Eggs are laid in stems, petioles, or veins of the leaves and nymphs commence hatching within 10-14 days except for the over-wintering eggs. The nymphs go through five instars of approx. 5-7 days.

In the field, adults may be taken during most months of the year, but the numbers are generally lower than with some other species. The peak population in the Australian Capital Territory occurs in April (Table 4) and this is so in certain areas in New South Wales. There is a wide range of host plants and the insects probably over-winter in the field principally in the egg stage, although females have been collected during almost every month in the year.

Parthenogenesis has been recorded in an agallian leafhopper by Black and Oman (1947) but no eggs from unfertilized *A. torrida* developed.

TABLE 4

COMPARATIVE DENSITIES OF *A. TORRIDA* AND *O. ARGENTATUS* IN LUCERNE FIELDS AT CANBERRA, A.C.T., 1947-48

Monthly totals from weekly surveys

Month	*A. torrida*	*O. argentatus*	Month	*A. torrida*	*O. argentatus*
November 1947	3	3000	May 1948	37	8
December 1947	10	1842	June 1948	10	1
January 1948	0	392	July 1948	2	0
February 1948	8	556	August 1948	0	0
March 1948	28	369	September 1948	1	29
April 1948	90	101	October 1948	3	85
			November 1948	3	351

(*d*) *Feeding Behaviour*

Feeding tracks of *A. torrida* on *Malva* were studied by the methods employed by Day, Irzykiewicz, and McKinnon (1952). A portion of petiole was exposed to 10 adults for 65 hr. In sections of this petiole 108 feeding tracks were identified; each insect made an average of about four tracks per day. Fifty-one per cent. of the tracks terminated in parenchyma, 10 per cent. in xylem, 34 per cent. in phloem, and 6 per cent. in both xylem and phloem. The feeding behaviour was thus very similar to that of *Eurinoscopus punctatus* Evans, but the tissue damage was less severe than that resulting from feeding by this species. *A. torrida* is thus well adapted to the transmission of a phloem-inhabiting virus.

VII. DISCUSSION

(*a*) *Evidence that the Virus has not Previously been Described*

It is apparent that the symptoms of rugose leaf curl virus on a variety of hosts are widely different from those produced by any previously described virus. Sukhov and Vovk (1947) described a leaf curl of tomato that is transmitted by an agallian leafhopper. It is undoubtedly more closely related to rugose leaf curl than any disease so far described. However, rugose leaf curl virus produced no symptoms on potato, pepper, or egg plant, all of which were hosts of the Russian virus. Furthermore, characteristic deep red and orange spotting of tomato fruits was a feature of the tomato leaf curl disease, but was not found in tomatoes infected with rugose leaf curl.

Somewhat similar symptoms on tomato were described by McLean (1935) in plants infected with tomato bunchy top in South Africa. This, however, is mechanically transmitted with ease, is normally transmitted by aphids, and has a different host range from rugose leaf curl.

The relationship of the new virus to the "mild clone" of lucerne witches' broom virus described by Helms (unpublished data) is not clear. On lucerne the symptoms are very similar, but on tomato they differ widely and Helms's virus was transmitted by dodder. However, *A. torrida* is widespread in lucerne fields in Australia and it seems very likely that it causes a disease in lucerne indistinguishable on symptoms alone from Helms's mild clone of lucerne witches' broom. Unlike *O. argentatus,* the proved vector of lucerne witches' broom virus, this leafhopper breeds on lucerne, and is therefore potentially of more significance as a disease vector, especially as the virus is transmitted transovarially.

(*b*) *Reasons for Choice of the Name "Rugose Leaf Curl"*

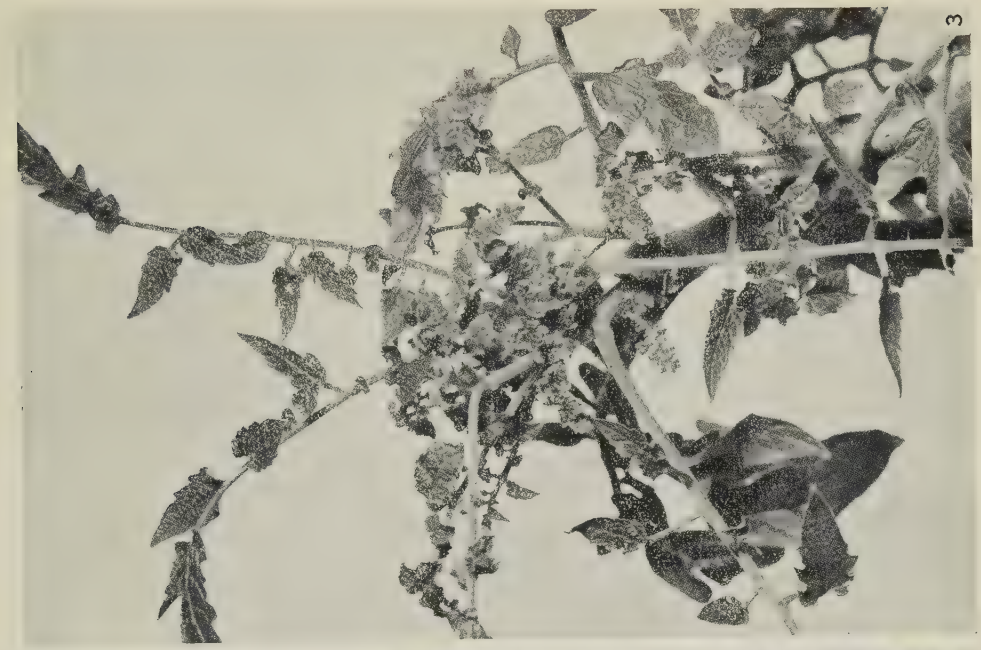
It is apparent from the description of symptoms and the comparisons made in the previous paragraphs that the disease described in this paper has not hitherto been reported. In naming the virus it is unsatisfactory to follow the usual course recommended by the Commonwealth Mycological Institute of prefacing the virus name with that of the main host. Symptoms of the new virus are very similar on a number of plants of economic importance and the name rugose leaf curl describes these fairly accurately.

(*c*) *Transovarial Infection in Leafhopper-borne Viruses*

Transovarial infection has been established in only four species of leafhopper (see Black 1953). In three of these, the vectors are agallian leafhoppers. This is in sharp contrast to the deltocephaline leafhoppers, where only one of over 30 species examined has been found capable of transmitting viruses through the egg. It is apparent that transovarial infection is a remarkable feature of viruses transmitted by some members of the Agalliinae. It is clear from the researches of Black (1950) and others that transovarial infection in leafhoppers is generally associated with multiplication of the virus in the insect, and it is likely that rugose leaf curl multiplies in *A. torrida.*

It has been noted by Fukushi (1940) that some individual leafhoppers, vectors of rice-stunt virus, although carrying virus during their entire life and passing the virus through the egg to their progeny, never transmit it to plants although confined throughout their life on susceptible species. Black (1953) also found that some female *Agalliopsis novella,* which failed to transmit clover club leaf, produced progeny carrying the virus. This may be so with *A. torrida* also, since on two occasions colonies of this leafhopper failed to transmit the rugose leaf curl virus to susceptible plants on which they were caged but the

RUGOSE LEAF CURL AND TRANSOVARIAL TRANSMISSION



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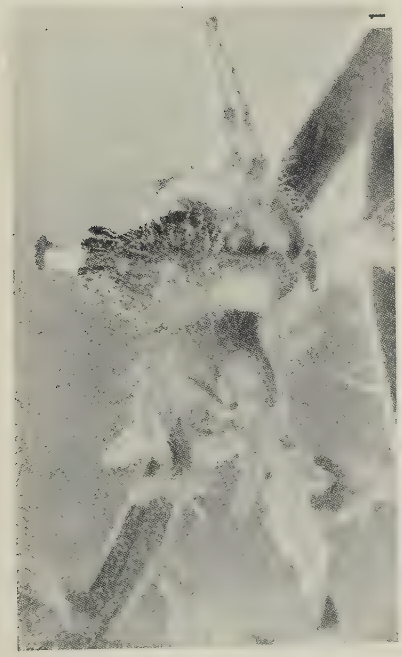

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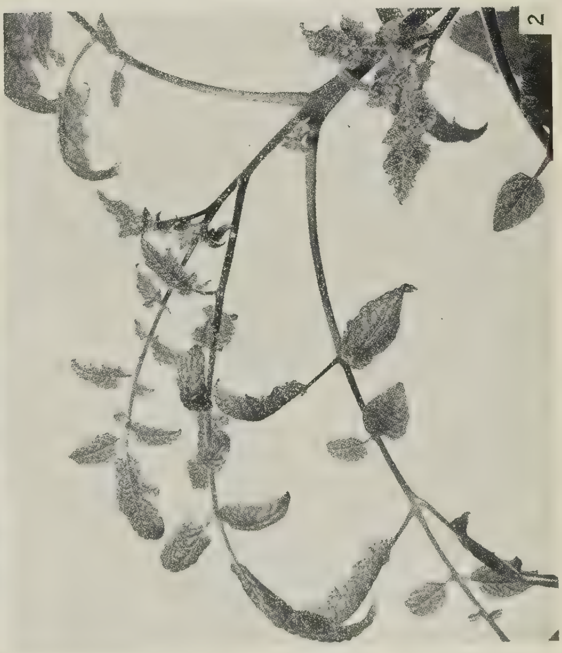

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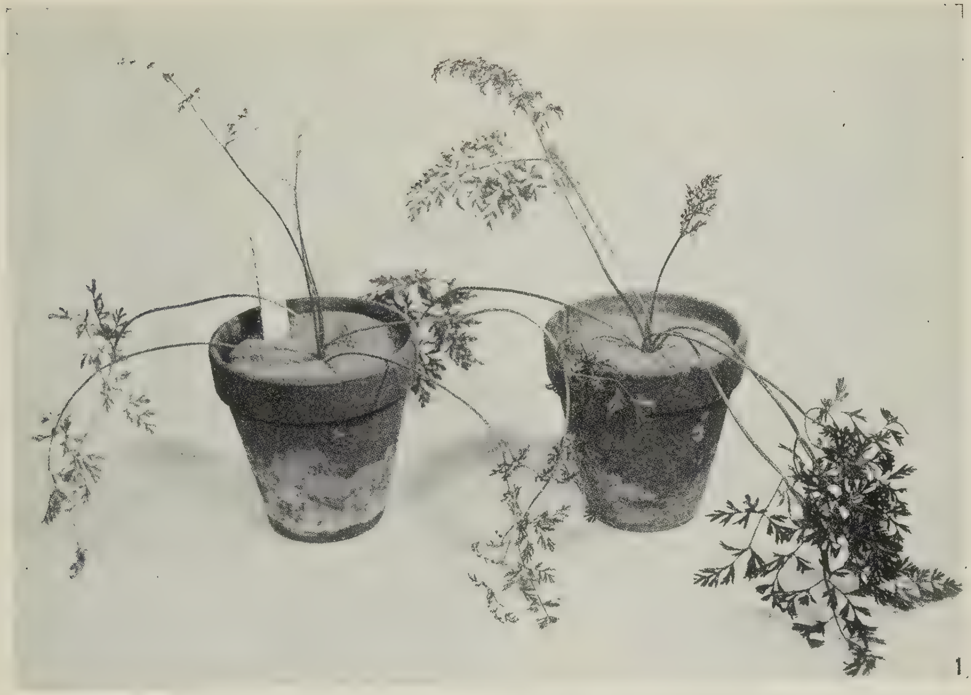
RUGOSE LEAF CURL AND TRANSOVARIAL TRANSMISSION



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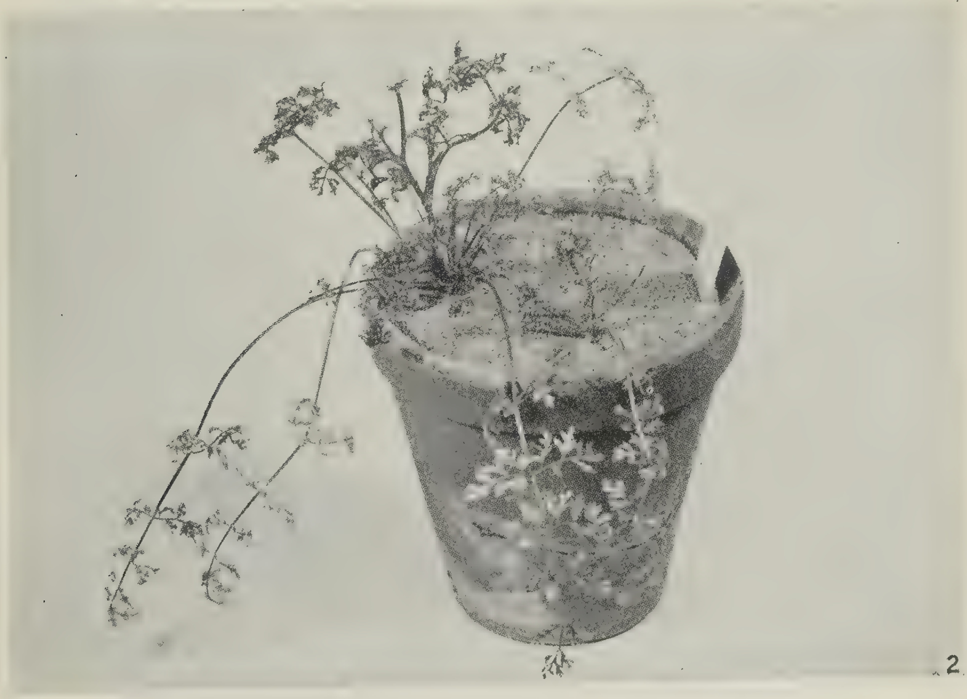

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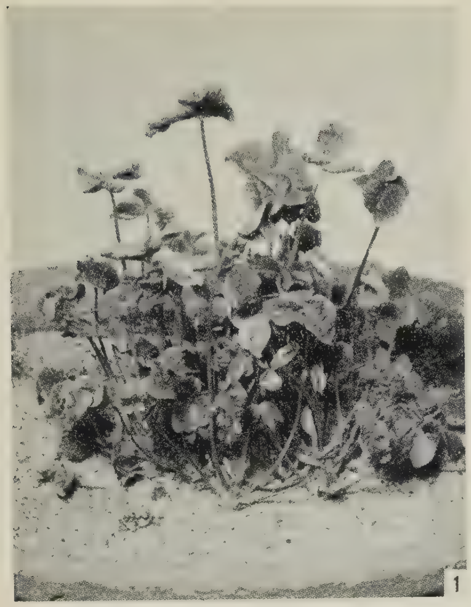
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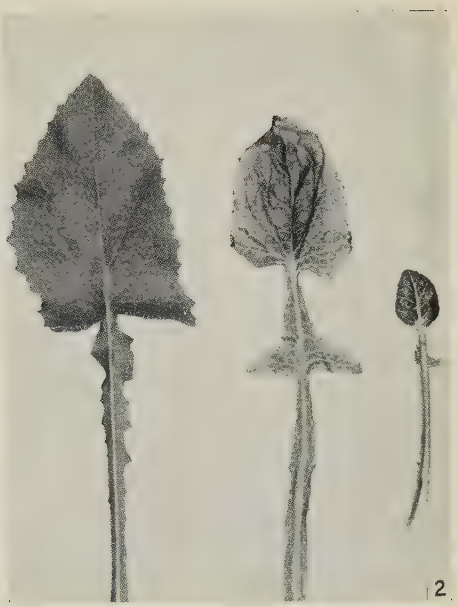
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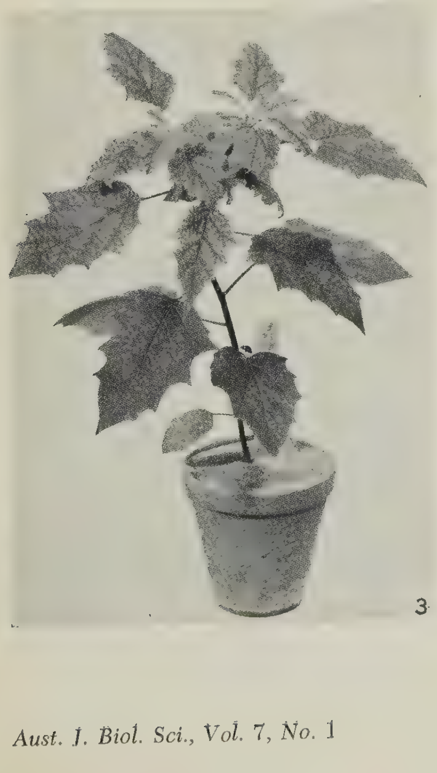
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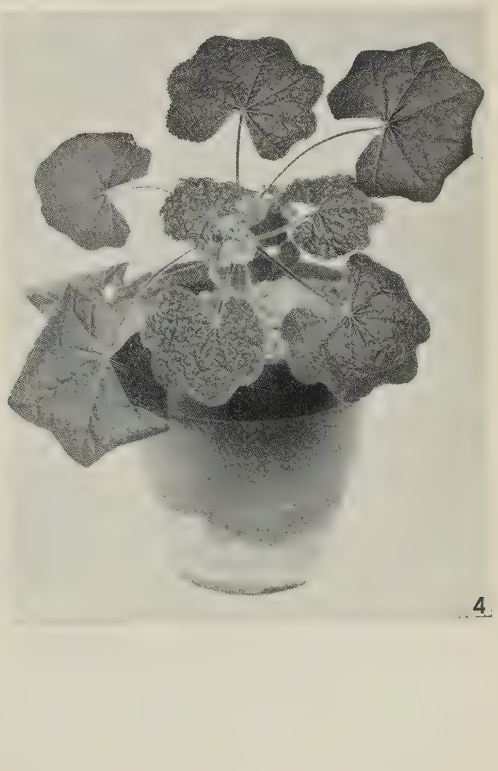
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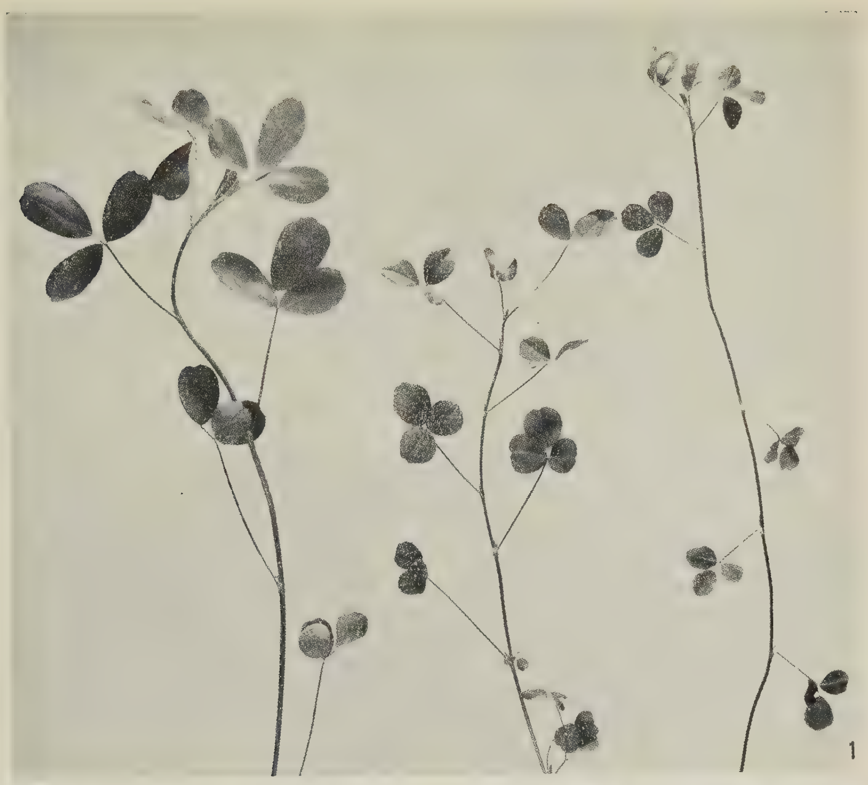
RUGOSE LEAF CURL AND TRANSOVARIAL TRANSMISSION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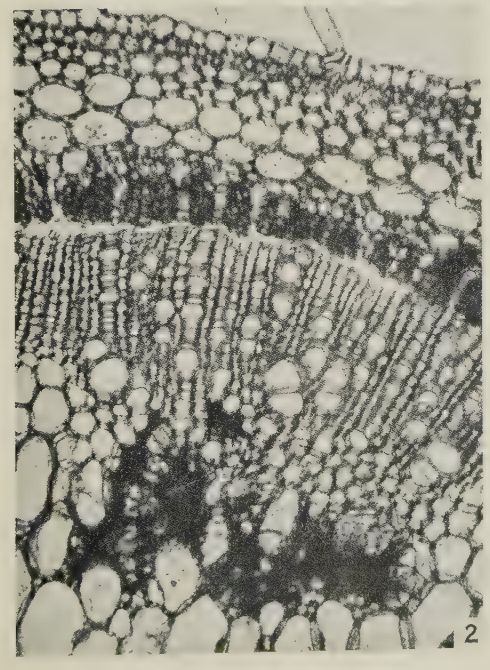
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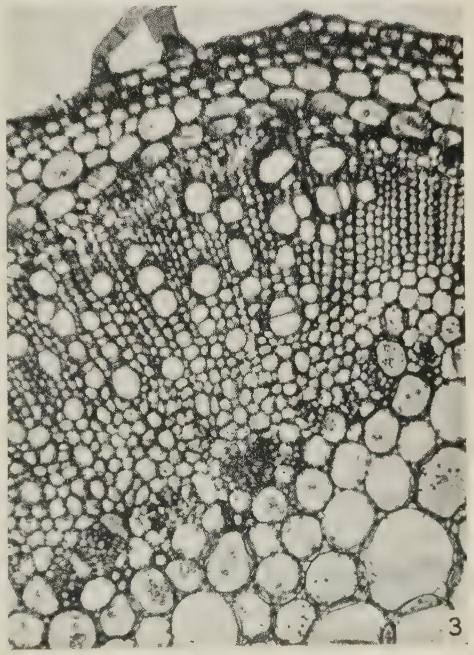
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virus was transmitted by later generations. The latent period of rugose leaf curl in *A. torrida* has not been studied because of the difficulty of obtaining a virus-free colony of insects. In both instances where it was considered that virus-free insects had been obtained, transmission occurred to control plants on which they were fed.

VIII. Acknowledgments

Thanks are due to many colleagues for advice in the course of this work, particularly to Dr. M. F. Day, Dr. C. Barnard, and Mr. H. Irzykiewicz. The photography is the work of Mr. D. Wilson. Specimens of the vector were received from Dr. E. Phillis, Mr. M. H. Wallace, and Mr. A. V. Hill. Dr. J. W. Evans confirmed the identifications and provided valuable details concerning the vector. The assistance of the Principal and staff of the Hawkesbury Agricultural College during field surveys is also appreciated.

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EXPLANATION OF PLATES 1-4

PLATE 1

Symptoms of rugose leaf curl caused by virus transmitted by *A. torrida.*

Fig. 1.—Green bud with protruding pistil from tomato plant in Figure 3.
Fig. 2.—Early symptoms on tomato.
Fig. 3.—Advanced symptoms on tomato.

PLATE 2

Symptoms of rugose leaf curl caused by virus transmitted by *A. torrida.*

Fig. 1.—Early stage of carrot compared with healthy plant.
Fig. 2.—Late stage in development on carrot.

PLATE 3

Symptoms of rugose leaf curl disease.

Fig. 1.—Fully developed symptoms of the virus on red clover (*T. pratense*).
Fig. 2.—Early symptoms on milk thistle, showing vein-clearing and rugosity.
Fig. 3.—Rugosity and marginal chlorosis on *D. tatula* (virus transmission by graft).
Fig. 4.—Symptoms on *M. parviflora* (virus transmitted by *A. torrida*).

PLATE 4

Fig. 1.—Comparison of symptoms of rugose leaf curl and lucerne witches' broom with healthy lucerne. From left to right: healthy; rugose leaf curl; and infected with witches' broom.
Figs. 2 and 3.—Transverse sections of tetraploid *P. floridana.*
Fig. 2.—Section of an infected plant showing hypertrophy and necrosis of phloem tissue.
Fig. 3.—Section of healthy plant of the same age.

THE RATE OF PRODUCTION OF THE PERITROPHIC MEMBRANE IN SOME INSECTS

By D. F. Waterhouse*

[*Manuscript received August* 20, 1953]

Summary

Feeding zygopterous (damsel-fly) nymphs produced peritrophic membranes at a rate of about three per day. Each membrane is secreted by the entire midgut epithelium and varies in length according to the degree of distension of the midgut by food. Starving nymphs evacuated empty membranes at a slower rate (about 1 per day) until death.

Larvae of the drone-fly *Eristalis tenax,* when transferred to fresh water, evacuated their continuous, tubular peritrophic membrane for some hours at 30°C at a rate of about 5 mm/hr. After 42 hr without access to food the rate dropped to about 2 mm/hr. The corresponding production rates for larvae in dilute, filtered liquid manure were 6 and 3 mm/hr respectively. Food passed through the alimentary canal of actively feeding larvae at a rate varying from 50 to 75 mm/hr and therefore travelled at a very much greater rate than that at which the peritrophic membrane was produced.

In blowfly larvae, removed from their food and kept at 30°C, the continuous peritrophic membrane was produced at a rate varying between 5 and 10 mm/hr for the first 24 hr. Food passed down the alimentary canal at about 75 mm/hr.

Well-fed adult earwigs discharged their peritrophic membrane at an average rate of about 1.6 mm/hr at 30°C. Food passed along the alimentary canal at this rate or slightly faster. At about 18°C fed individuals produced their membrane at about 0.25 mm/hr. During starvation at 30°C the rate of membrane production fell to 0.6 mm/hr.

I. Introduction

Although much has been written on the occurrence of the peritrophic membrane (PM) in insects and on its mode of production, there is very little information available on the rate at which it is produced or on how this rate is influenced by the intake of food.

The PM is produced in some insects by a ring of cells at the anterior end of the midgut (type I) and in others by the delamination of one or more layers from the surface of the midgut epithelium (type II). Whatever the method of production, the PM appears to be present continuously in most insects which produce it, although in some, e.g. adult mosquitoes (Waterhouse 1953), it has only been detected after feeding. Since fragments of membrane are present in the faeces of most insects it follows that the PM is either produced continuously or at intervals and in either case is passed on with the food.

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Where the membrane is produced at the anterior end of the midgut it is generally thought to be passed on at about the same rate as the food. This is suggested by observations, such as those on the tsetse fly, *Glossina* (Wigglesworth 1929), in which the PM in the freshly emerged adult is ragged and discontinuous. Immediately the first meal is ingested the PM is perfectly formed and extends to the limits of the ingested blood. Further, the data on the development and distribution of trypanosomes within the fly can be explained only if the PM is not penetrated by trypanosomes and if it extends continuously as far as the middle of the hindgut before infected blood has reached this level (Hoare 1931; Yorke, Murgatroyd, and Hawking 1933).

The only numerical data on the rate of production of the type I PM is that of Aubertot (1932*a*) who observed that a single *Eristalis tenax* (L.) larva, placed in fresh water for 17½ hr, produced a PM at the rate of 6.1 mm/hr.

There is very little more known on the rate of formation of the delaminated type of PM, where the information required is the frequency with which the layers comprising the PM are formed. Rengel (1903) reported that at least five to six membranes were delaminated by wasp larvae (*Vespa*) and an even larger number by honey-bee larvae. The rate could be measured because the food residues are retained in the larval midgut until pupation. As food continues to be ingested the innermost membranes burst, whereas the outer, larger, more recently formed membranes remain intact. It is possible, therefore, to make an estimate of the number of layers of membrane at various stages of larval development. Aubertot (1932*b*) recorded that a single starving *Aeschna* (dragonfly) larva produced PM's at the rate of two per day for 4 days. In this instance the number of membranes produced could be recorded since, at intervals, the midgut contents (together with their enveloping PM) are passed rapidly down the hindgut and excreted.

The principal difficulty in measuring the rate of production of the PM, except in such specialized forms as larval Hymenoptera, is to recover it in sufficiently undamaged form from the faeces. Although the relatively tough type I membrane is produced by many insects it is frequently broken into small pieces in the hindgut before discharge. Thus, in adult blowflies the rectal papillae carry backwardly directed spines which break up the membrane so effectively that only fragments can be recovered from the faeces. In insects which produce the delaminated type of PM the faeces are frequently solid or semi-solid. When the food residues, enclosed in the PM, are passed into the hindgut (e.g. *Locusta, Periplaneta*) the PM is fragmented during the formation of the faecal pellets, so that it is difficult to relate the length of the faecal pellet to the length of PM originally enclosing the corresponding mass of food in the midgut. Furthermore this gives no direct information on the frequency of delamination from the surface of the midgut epithelium.

The formation of solid or semi-solid faecal pellets is often related to the insects' need to conserve water. It is noteworthy, for example, that the PM is discharged in a relatively undamaged condition by many aquatic or semi-aquatic insects and it is these insects which appear to provide the best material for measuring its rate of production. Another advantage in using aquatic larvae is that the task of recovering for examination the transparent, almost invisible PM is greatly simplified if it remains suspended in water. For these reasons it was decided to use two aquatic insects (nymphs of *Ischnura heterosticta* Burm. (Odonata), larvae of *Eristalis tenax* (L.) (Diptera : Syrphidae)) and three insects which feed on moist food (larvae of *Lucilia cuprina* (Wied.), *Calliphora augur* (F.), and *C. fallax* Hardy: Diptera : Calliphoridae). The earwig *Labidura truncata* Kirby was also used. Dermaptera produce the inner portion of their PM from cells at the level of the oesophageal invagination and the outer layers by delamination from the general midgut epithelium. This then represents a type intermediate between the Odonata and Diptera examined. Information was particularly desired on the rate of production (or delamination) in feeding and starving insects and on the relative rates of passage of food and PM along the alimentary canal.

II. Methods and Material

(*a*) *Odonata*

Nymphs of several species of Odonata (mainly belonging to the sub-order Zygoptera (damsel-flies)) were collected from a stream and kept individually in small glass vessels in fresh water to a depth of 5 or 6 mm. The most abundant species was *Ischnura heterosticta* Burm. (Coenagriidae), although *Austrolestes cingulatus* Burm. (Lestidae) and *Austroagrion cyane* Selys. (Coenagriidae) were present in some batches of nymphs. All species of Zygoptera appeared to behave similarly. The nymphs were either starved or offered an abundant supply of mosquito larvae (*Aedes aegypti* L. or *A. notoscriptus* Skuse). A multilayered PM varying in thickness from about 0.5 to 2.0 μ is formed in the midgut and is retained therein for some hours before being passed on *in toto* to the hindgut. Up to eight layers have been distinguished in membranes from feeding nymphs, although three or four is a more usual number. However, it is not possible to estimate the number of layers at all accurately. In fact it is generally only when some of the component layers become fortuitously separated that it is possible to make out the individual layers at all clearly. In Odonata the rectum has an important respiratory function and the membrane is only permitted to remain in the hindgut for a relatively short time before being evacuated and hence is discharged in an undamaged state.

Both number of multilayered membranes produced and their length were recorded at regular intervals and, in addition, a record kept of the consumption of mosquito larvae in order to indicate whether the nymphs were in an actively feeding condition.

(*b*) *Diptera*

Larvae of *Eristalis tenax* (L.) (Syrphidae) were obtained from liquid horse manure both from a laboratory culture and from the field. Larvae ranging in length from 10 to 15 mm (to the base of the respiratory siphon) were used in most experiments, although slightly smaller or larger larvae were employed occasionally. These lengths are only approximate, since it is very difficult to make measurements with any accuracy when the larvae are actively extending and contracting their bodies. The larvae were washed in fresh water and placed in squat 10-ml beakers containing water or coloured liquid manure to a depth of about 1 cm. Very few larvae escaped so long as there was sufficient fluid to cause them to swim and provided that the diameter of the container was greater than the length of the body.

The tubular continuous PM projected from the anus as it was excreted, pieces of variable length (from a millimetre or so to up to 60 mm) being broken off intermittently and fortuitously by larval movements. For measurements of rate of production, pieces of extruded membrane which had not been detached by the larvae were broken off as close as possible to the anus. Care had to be exercised to prevent several additional millimetres of membrane being pulled out of the anus in this process. The length of the pieces of membrane was measured under water, using a pair of adjustable dividers and a magnification of 6.6×. There were naturally errors in estimating the length of the fragments of PM, since it was difficult to get them to lie straight. Where several lengths from a single larva were measured at the same time it was found convenient to record each by pricking it on a ruled index card and to measure the total length produced. This procedure reduced the error in estimating fractions of a millimetre. The total lengths of membrane were recorded to the nearest 0.5 mm.

Almost fully grown third-instar blowfly larvae (*Lucilia cuprina* (Wied.), *Calliphora augur* (F.), and *C. fallax* Hardy) were removed from liver and, after washing, transferred to excavated blocks containing a few drops of water. The top of each block was covered with a "Perspex" lid in which several holes 1 mm in diameter had been bored. The larvae carried a film of water with them as they moved and this soon sealed the lid to the excavated block. The holes in the lid, however, enabled an adequate supply of oxygen to reach the larvae. The lengths of membrane produced were measured as for *E. tenax*.

(*c*) *Dermaptera*

Specimens of *Labidura truncata* Kirby were collected in the field and maintained individually in glass-covered excavated blocks. They lived well on "Farex*" moistened with water and this food resulted in a humidity approaching saturation being maintained in these containers, which prevented the faeces from drying out. The faecal pellets were transferred to water for examination. The pellets varied from a single short piece of membrane surrounding food residues to a group of half a dozen or more such pieces, some lying longitudinally and others transversely to the hindgut axis. Each of these pieces is detached from the remainder of the membrane in the hindgut just anterior to the rectum. When the pieces of membrane had been crumpled during passage through the hindgut, or if they were lying transversely in the faecal pellet they were carefully teased out and laid end to end before measuring. These measurements were undoubtedly subject to some error because it is not possible to reconstitute with a high degree of accuracy several dozen pieces, mostly

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[*] A proprietary baby food: Glaxo Laboratories (Aust.) Pty. Ltd.

varying from 0.5 to 1.5 mm in length. However, it is believed that the errors involved did not materially affect the conclusions reached.

TABLE 1

RATE OF PM PRODUCTION IN FED ZYGOPTEROUS NYMPHS

	No. PM's Produced		*A. aegypti* Larvae Eaten 0-48 Hr
	0-24 Hr	24-48 Hr	
1	4	3	7
2	4	4	13
3	4	2	4
4	3	4	12
5	3	4	6
6	2	3	7
7	5	2	10
8	3	4	6
9	3	3	9
10	3	2	4
Total	34	31	71

III. RESULTS

(*a*) *Zygoptera*

(i) *Rate of PM Production in Fed Nymphs.*—Ten nymphs varying in length from 10 to 17 mm (excluding the anal gills) were continuously supplied with *A. aegypti* larvae. The results (Table 1) show that, in these actively feeding nymphs, two to five multilayered membranes were produced per day with an average of 3.3 per nymph per day. Other observations also indicate that the normal daily production is usually in the vicinity of three membranes per nymph per day, although rates as low as 1.5 per day have been recorded (Table 2).

TABLE 2

INFLUENCE OF FEEDING AND STARVATION ON RATE OF PM FORMATION IN ZYGOPTEROUS NYMPHS

Series	No. Larvae	Nutrition	No. Days	PM's/Day
1	7	Fed	5	1·5
	6	Starved	4	1·2
	3	Fed	6	1·9
2	6	Starved	5	0·8
	3	Fed	7	2·7
	2	Starved	3	1·3

Although the average figures indicate that a membrane is discharged every 8 hr the intervals between the expulsion of successive membranes is rather irregular. The shortest interval recorded for fed nymphs was 2 hr.

(ii) *Effect of Starvation on Rate of PM Production.*—Two series of 10 nymphs were set up in individual containers and subjected to alternating periods of feeding and starvation, each period lasting several days (Table 2). The number of nymphs finally considered was less than the original 20. This is because figures for nymphs which moulted, died, or emerged during any one period were excluded from the records.

Table 3

RELATIVE LENGTHS OF PM'S AND MIDGUT OF ZYGOPTEROUS NYMPHS

Length of Midgut (mm)	Length of PM's (mm)
4·5	6·5
2·5	3·0
3·0	3·0, 3·0, 3·0 (empty), 5·5, 5·5 (full)
3·5	4·0, 4·0, 4·5, 4·0, 3·0, 2·5
2·5	2·5, 2·0

In both series the frequency of PM discharge was lower in starved than in fed nymphs. Once the starving phase had been initiated and the food residues in the digestive tract eliminated, the nymphs produced transparent empty membranes. There was no consistent downward trend in the rate of production over any one period of observation.

Table 4

LENGTH OF PM'S DISCHARGED BY INDIVIDUAL ZYGOPTEROUS NYMPHS

	Length of PM (to nearest 0·5 mm)	Range	No. PM's
1	4·5, 3, 6, 6·5, 4·5, 7, 9, 4·5	3-9	8
2	5·5, 7·5, 5, 8, 5, 8, 7, 7, 4, 4, 4, 4, 4·5, 7, 3·5	3·5-8	15
3	4, 7, 7, 4, 3·5, 6	3·5-7	6
4	5·5, 6·5, 6·5, 6·5, 8, 6·5, 6, 7, 7, 6·5, 5·4, 3·5	3·5-8	12
5	3, 5, 5, 4, 4·5, 4·5, 5, 4	3-5	8
6	7, 7, 5, 5·5, 7·5, 5, 5, 5, 8, 5·5, 3, 6·0	3-8	12
7	4·5, 5, 9, 9, 5·5, 6, 6·5, 6, 6, 4·5, 5, 2·5	2·5-9	12
8	4·5, 4, 4, 3·5, 3·5, 3, 2·5, 2	2-4·5	8
9	3, 4, 4·5, 4·5, 3·5, 4·5, 3	3-4·5	7
10	2, 3, 3, 2·5, 2·5	2-3	5

(iii) *Relation of Length of PM to Length of Midgut.*—Five nymphs were dissected after they had produced one or more membranes and the lengths of the PM's and midguts measured (Table 3). Records were also kept of the lengths of successive PM's produced by 10 nymphs (Table 4).

It is clear from these figures that there is a very considerable variation in the lengths of successive PM's produced by the same larva. Furthermore, the lengths may be considerably greater than, equal to, or slightly less than the length of the midgut as measured on dissection. This variation is undoubtedly due to the great capacity of the midgut for distension. The lengths of the PM's produced by starving nymphs were found to be relatively constant and to approximate closely to the length of the midgut. However, since food is taken intermittently (even if it is continuously available) and since the size of the meal varies enormously (from a single small mosquito larva to two or three large larvae) the distension of the midgut varies accordingly and membranes of variable length are produced as a result.

(iv) *Rate of Passage of Food.*—Some finely divided food material was observed to have reached the posterior end of the midgut of a previously starved nymph 4 or 5 min after a mosquito larva had been caught. However, the main bulk of a meal is frequently retained in the crop for some hours before being passed on to the midgut. The average time of passage of food through the alimentary canal is in the vicinity of 8 hr. As mentioned earlier, the shortest time recorded between the evacuation of successive membranes was 2 hr, so that food on this occasion passed through midgut and hindgut in this period.

(*b*) *Anisoptera*

A few observations on unidentified dragonfly nymphs indicated that these behaved in a fashion similar to damsel-fly nymphs. Membranes, which varied rather widely in length, were produced at a rate of about 1.8 per day. The most rapid rate of production observed was two membranes within 5 hr.

(*c*) Eristalis *Larvae*

E. tenax larvae do not possess a storage crop to maintain for some time a continuous supply of food to the midgut when larvae are removed from their food medium. Under natural conditions they ingest food continuously. When placed in fresh water therefore, as in some experiments, the rate of passage of food and the rate of production of the PM might be expected to be influenced by this somewhat unnatural environment.

(i) *Rate of Production of the PM*

(1) *In Fresh Water.*—The rate of production of the PM was investigated at 2-hr intervals for 6 hr after larvae had been transferred to fresh water.

As can be seen in Table 5, there was a great individual variation, not only in the total length of PM produced (from 11.0 to 47.0 mm) by the various larvae over the 6-hr period, but also in the lengths produced during any 2-hr period. Undoubtedly one of the factors involved in the very variable rate of production by the same larva is that the PM is coiled upon itself in various regions of the gut. Lengths of membrane longer than usual may then be discharged at irregular intervals either naturally or when the larvae are stimulated by handling. During the first 2-hr period the larvae produced lengths of PM at the rate of

5.3 mm/hr. The rate dropped during the second 2-hr period to 3.7 mm/hr and rose during the final 2-hr period to 5.4 mm/hr, with an overall production rate of 4.8 mm/hr. The greatest individual production rate was observed during the 4-6-hr period when a single larva produced a 29-mm length of membrane.

In spite of the variability encountered it is clear that the PM is produced continuously and at a fairly constant rate, at least for the first 6 hr after removal from food.

TABLE 5

RATE OF PRODUCTION OF PM BY *E. TENAX* LARVAE SUSPENDED IN FRESH WATER AT 30°C FOR 6 HR AFTER REMOVAL FROM LIQUID MANURE

	Larval Length (mm)	Length of PM Produced (mm)			
		0-2 Hr	2-4 Hr	4-6 Hr	Total for 6 Hr
1	10	7·5	0	6	13·5
2	12	10	11·5	13·5	35·0
3	13	3·5	5	2·5	11·0
4	13	5·5	4	21	30·5
5	13	6	2	3	11·0
6	14	19	5	15	39·0
7	14	8	9	3	20·0
8	15	7	2·5	29·0	38·5
9	16	24·5	5	9·5	39·0
10	16	14·5	12	13	39·5
11	16	9·5	7	3·5	20·0
12	16	2·5	13	5	20·5
13	17	0	11·5	5	16·5
14	17	17·5	8	13	38·5
15	17	14	19·5	13·5	47·0
16	18	7·5	5	17·5	30·0
17	19	24	6	9·5	39·5
Totals		180·5	126·0	182·5	489·0
Rate per larva per hour		5·3	3·7	5·4	4·8

(2) *In Filtered Liquid Manure.*—It was necessary to determine whether the rate of production was appreciably lowered in the above experiment because no fresh food was being ingested by the larvae during the period of test.

Larvae were therefore set up in diluted, filtered liquid manure to determine whether the production rate was greater under these conditions than in fresh water (Table 6). The suspending medium had to be free from solids to enable all PM fragments to be recovered.

The results clearly indicate that the average production rate is higher in larvae living in liquid manure than in water. The drop in the rate of PM production between 2 and 4 hr and its subsequent rise between 4 and 6 hr (also recorded in Table 5) are at present unexplained. The production rate fell gradually after the first 6-hr period, but it is clear that the larvae continued to secrete a membrane over the entire experimental period. The greatest individual production rate recorded in these experiments was 36 mm between 4 and 6 hr for a larva in fresh water.

It is unfortunately not possible to determine whether the PM production rate is still higher when larvae are feeding in their more normal semi-solid medium, because of the difficulty of recovering all lengths of peritrophic membrane. However, from the fact that the rate of production is not very different for the first 10 or 15 min after removal from their natural food than for the next couple of hours, it seems unlikely that the average production rate would exceed 10 mm/hr. Arguing similarly, the maximum production rate might possibly be 30 mm/hr instead of the maximum of 18 mm/hr observed.

TABLE 6

PRODUCTION OF PM BY 10 *ERISTALIS* LARVAE SET UP IN FRESH WATER AND IN DILUTE FILTERED LIQUID MANURE

	Fresh Water					Dilute Filtered Liquid Manure				
	0-2 Hr	2-4 Hr	4-6 Hr	6-24 Hr	24*-48 Hr	0-2 Hr	2-4 Hr	4-6 Hr	6-25 Hr	25-49 Hr
Total length produced (mm)	91	72	100	492	429	122	56	122	698	611
Rate per larva per hr (mm)	4·6	3·6	5·0	2·8	2·2	6·1	2·8	6·1	3·7	2·8

* Two larvae died during this period and were excluded from the calculations.

(ii) *Rate of Passage of Food*

The most rapid rate of food passage from mouth to anus observed at 30°C was 80 min for several larvae transferred to liquid manure containing finely powdered animal charcoal. One of these larvae was approximately 11.5 mm long and had a total gut length of 66 mm. Two other larvae were 14 mm long and each had a gut length of approximately 100 mm. The food, therefore, passed through the alimentary canal in these larvae at a rate between 50 and 75 mm/hr.

In a number of other larvae tested either with charcoal or finely divided Prussian blue as markers, food had passed through to the anus in 85-120 min. On the other hand in some larvae the coloured food had only travelled as far as the middle or end of the midgut after 2 hr and in others it had not quite reached the end of the hindgut after 24 hr. There are evidently variations in the rate of passage of food which may be due to individual variation between larvae or related to differing physiological states.

When active larvae were suspended in fresh water the majority had discharged all food residues from the gut in 6 hr or less.

(iii) *Relative Rates of Food Passage and Membrane Production*

When the deduced maximum rate of PM production of about 30 mm/hr is compared with an observed rate of passage of food of 50-75 mm/hr it is apparent that the food must often pass down the alimentary canal at an appreciably greater speed than the PM. This conclusion is supported by many observations of larvae which have caused considerable fouling of the fresh water in which they have been placed without extruding more than a few millimetres of membrane during the same period. Furthermore, when larvae had been transferred from liquid manure + carbon to liquid manure + congo red, particles of carbon could be detected within the PM more than 1 cm behind the advancing front of the congo red-stained food, suggesting that they had been left behind the main mass of carbon-marked food. It was observed in these experiments that the PM was permeable to congo red, which is what might have been expected from similar results with adult *Calliphora* (von Dehn 1933).

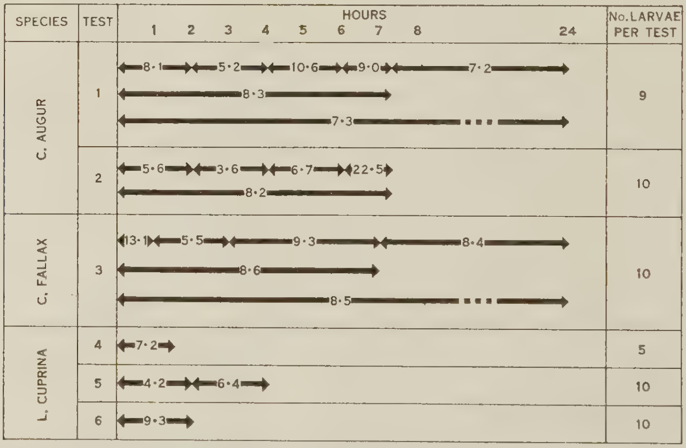

Fig. 1.—Chart showing the average rate of production of PM (mm/hr) by calliphorid larvae at 30°C. The figure within a segment of horizontal line is the average rate for the period of hours (after removal from food) covered by that segment.

(*d*) *Blowfly Larvae*

(i) *Rate of Production of the PM.*—Figure 1 summarizes the results obtained for rate of PM production with three species of blowfly larvae. Although there was considerable individual variation in production rates, particularly over shorter periods of time, the average rate for all three species generally fell within the range 5-10 mm/hr. Rates for the first 7 (or 7¼) hr for the two *Calliphora* species varied from 8.2 to 8.6 mm/hr. There is no reason to believe that this rate is appreciably less than in feeding larvae. The maximum rate observed was 67 mm in 1¼ hr (from 6 to 7¼ hr) for a *C. augur* larva. This was almost certainly due to the discharge of stored-up PM produced over a much longer period, since this larva had discharged membrane at only 4 mm/hr for the previous 6 hr. It is clearly necessary to measure production rate over a period long enough to average out such individual variations in discharge rate, but not long enough to extend much into the period when the production rate starts to diminish. The temporary reduction in rate of discharge between 2 and 4 hr, already noted for *Eristalis,* also occurred in these experiments.

(ii) *Rate of Passage of Food.*—Almost fully fed third-instar *L. cuprina* larvae were transferred to finely ground liver coloured with solid Prussian blue and maintained at 30°C. Measurements for these larvae were: weight 34 mg, total length 10 mm, foregut 3 mm, midgut 75 mm, hindgut 35 mm. In those which commenced to feed immediately after transfer, coloured food had travelled in 10-15 min some 40 mm to reach the middle region of the midgut. This region has, for a short distance, a greater diameter than the PM, which consequently coils up upon itself several times. If recently ingested food already occupies the middle region of the midgut coloured food is temporarily held in the anterior midgut. Coloured food usually passes no further than the mid midgut until some 50-60 min after transfer, when it moves down through the posterior midgut and hindgut to reach the anus about 90 min after ingestion. The larvae all had full crops when transferred and coloured food usually moved straight past the crop into the midgut. Occasionally, however, a small amount of this coloured food found its way into the upper third of the crop.

It is clear from these figures that the rate of passage of food along the digestive tract is many times greater than that at which the PM is produced, a result already noted for *Eristalis* larvae.

(*e*) *Dermaptera*

(i) *Rate of Production of the PM.*—Individual *Labidura truncata* varying in length from 13 to 21 mm (tip of head to base of forceps) were maintained at 30°C and supplied daily with fresh food. No important differences were observed in rate of PM production between males, females, and large nymphs.

In a typical experiment 2804 mm of membrane were produced in 1748 'earwig hours,' an average rate of 1.6 mm/hr. As an indication of the variation in production rate, the average rate for the first 958 hr of this period was 1.7 mm/hr and, for the second period of 790 hr, 1.4 mm/hr. Figures for the second period are shown in Table 7. The fastest individual rate over a 24-hr period was 3.4 mm/hr (for a nymph 13 mm long) and the slowest was 0.4 mm/hr (for a female 19 mm long). Values approaching these were, however, not common.

At room temperature (15-18°C) activity was restricted and feeding intermittent. Over a 13-day period a PM was produced at a rate of 0.25 mm/hr.

(ii) *Effect of Starvation on Rate of PM Production.*—Individuals were supplied with drinking water only (in small glass tubes) and maintained at 30°C.

For 48 hr before starvation commenced they produced PM's at a rate of 2.2 mm/hr. In the first 48 hr of starvation the rate fell to 0.9 mm/hr and levelled out at 0.58 mm/hr for the following 10 days. No deaths occurred during this period and the membranes produced were devoid of contents.

(iii) *Rate of Passage of Food.*—A starved *Labidura* commences to feed immediately food is presented. Food passes almost immediately to the posterior end of the crop and, at 30°C, within 30 min it has generally reached the end of the midgut. By 3 hr it may reach the rectum and faecal pellets may be produced from 4 hr onwards.

Table 7

PM PRODUCTION (MM) AT 30°C IN CONTINUOUSLY FED *LABIDURA*

Period (hr)	Length (mm) of Membrane Produced				
	1	2	3	4	5
24	60	30	34	34	61
24	22	30	39	14	23
7	13	8	10	2	12
24	33	58	30	24	59
24	32	39	34	56	31
24	16	17	15	29	11
24	19	31	32	81	14
7	12	9	11	14	14
158	207	222	205	254	225

The length of the alimentary canal varies somewhat according to the degree of distension by food. For individuals measuring 15-20 mm from the tip of the head to the base of the forceps the engorged foregut measures 15-19 mm in length, the midgut 4-6 mm, and the hindgut 7-9 mm. Assuming immediate passage of food into the midgut in starved individuals, passage of food along the 4-6 mm midgut length took half an hour (equivalent to a rate of 8-12 mm/hr). The most rapid recorded rate of PM production (for 24 hr) was 3.4 mm/hr, which suggests that the food in previously starved animals may be passed on more rapidly than the PM.

Rate of food passage in well-fed *Labidura* is appreciably slower than in previously starved individuals. Coloured faeces were not infrequently passed 7-8 hr after stained food was offered, a minimum of 9 mm of PM being discharged in this period. From these figures it appears that food normally passes through the alimentary canal at about the same rate as (or slightly faster than) that at which the PM is produced.

IV. Discussion

Figures are now available to support the conclusion that the type I PM is produced continuously and at a rate which is higher in actively feeding than in starving insects.

By contrast, the midgut epithelium evidently produces the lamellae of the type II membrane intermittently, periods of secretion alternating with periods when the epithelial cells are elaborating further precursor materials. The intervals between periods of delamination are shorter, in the zygopterous nymphs examined, in fed than in starving insects. Unfortunately the individual lamellae are not only insufficiently separated, but are also too fine to be counted accurately, so that the frequency of each delamination has not been determined. It appears to be once every hour or two.

Evidence that the PM is produced by the entire midgut epithelium in zygopterous nymphs follows from the observation that the length of the PM corresponds with the length of the midgut. Only if the entire midgut produced the PM would its length vary in accordance with the varying degrees of midgut distension, the latter depending upon the size of the meal ingested.

A somewhat unexpected observation was that food passes down the digestive tract in the fly larvae examined much faster than the rate at which the PM is produced. The rate of passage of food must be governed to a certain extent by the length of the alimentary canal to enable at least a minimum time for digestion and absorption to occur. The rate is thus considerably less in *Drosophila* larvae where, however, food also passes from mouth to anus in less than 2 hr (Bowen 1951). The more rapid passage of food than membrane results in some food particles being left behind the main mass moving within the PM. With a rate of food passage of the order of 75 mm/hr it is possible that the PM does provide a useful measure of protection for the midgut epithelium which, in the larvae of many Diptera, is notable in that it is not replaced at moulting as in most insects. Furthermore, the continuously produced PM ensures that all food residues are passed down the digestive tract at a rate not less than that at which the PM is formed. In the absence of a PM it is probable that some residue would remain for relatively long periods near the surface of the midgut cells. That this may happen to materials which have passed out through the PM is shown by observations on *Drosophila* larvae (Bowen 1951). Much of the radio barium added to their diet may be held for 10-20 hr in the space between the PM and the anterior midgut epithelium, and no ^{140}Ba was found in this space in the lower regions of the midgut. The fact that the fluid filling this space (containing as it does the products of digestion) moves so slowly in relation to the rapidly moving contents of the PM must greatly increase the efficiency of absorption.

V. ACKNOWLEDGMENT

I am indebted to Mr. R. Dobson for identifying the Odonata.

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THE THERMAL DESTRUCTION OF RIBOFLAVIN

By K. T. H. FARRER* and JOAN L. MACEWAN*

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Summary

The thermal destruction of riboflavin in buffer solutions from pH 0.5 to 12.0 has been studied by means of fluorimetric analysis and the application of the first-order reaction and Arrhenius equations. The buffer solutions used were a modified universal buffer (phosphate, borate, acetate), McIlvaine's citric acid-phosphate buffer, veronal-HCl, and glycine-NaOH.

Riboflavin in concentrations of 5 μg/ml is completely stable for 1 hr from pH 1.3 to 6.5 at 80°C, from pH 1.7 to 5.5 at 100°C, and from pH 2.0 to 5.0 at 120°.

Destruction at the higher pH's follows the first-order reaction and the Arrhenius equation relating rate of destruction to temperature. There is also a linear relation between the logarithm of the concentration of riboflavin and k, the rate constant.

At pH values below 7, the relation between log k and pH is roughly linear, but over the whole range studied kinetically (pH 5.0 to 9.5), and especially above pH 7.5, there is clear evidence of the varying catalytic effect of different buffer anions on the rate of destruction.

It has been shown by paper chromatography that the products of the thermal hydrolysis of riboflavin are ammonia, urea, 1,2-dihydro-6,7-dimethyl-2-keto-1-D-ribityl-3-quinoxalinecarboxylic acid, and an unidentified compound giving a weak mauve fluorescence in ultraviolet light.

Destruction of riboflavin at the very low pH values is a reversible reduction rather than a hydrolysis, as most of the fluorescence lost can be regenerated by aeration. Chromatography of these solutions yielded only riboflavin.

I. INTRODUCTION

Since the days when 'water soluble B' was first separated into heat-labile B_1 and heat-stable B_2, riboflavin has been considered to be stable to heat, at least under the conditions to which foodstuffs are exposed.

It is known that riboflavin is not stable in alkaline solutions (Chick and Roscoe 1930; Kuhn and Moruzzi 1934), and its alkaline hydrolysis has been described (Surrey and Nachod 1951). Loy, Haggerty, and Combs (1951) have recently recorded the loss of riboflavin on heating for 1 hr at 121-3°C at pH 0-13. They showed that, under these conditions, there is a rapid destruction below pH 1 and above pH 5.4.

This paper presents a kinetic study of the thermal destruction of riboflavin, records the catalytic effect of various buffer salts, temperature, and riboflavin concentration, and describes the products of the reaction. While it was being

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completed, a report of the work of Shimizu (1950) has appeared. Unfortunately, the original reference is not available in this country, but it would appear that similar results were obtained at much lower temperatures. The different effects of various acids and buffer salts on riboflavin destruction in solutions of increasing pH were noted, and destruction was found to be greater at low concentrations.

The chromatography of riboflavin and its photodegradation products has been described by Hais and Pecakova (1949). Good separation of the various compounds, riboflavin, lumiflavin, and lumichrome, as well as two unidentified spots, was obtained using butanol-acetic acid-water (3:4:7). These authors studied only the products of the irradiation of riboflavin.

According to Surrey and Nachod (1951), the most likely products of the thermal destruction of riboflavin at high pH values are urea and 1,2-dihydro-6,7-dimethyl-2-keto-1-D-ribityl-3-quinoxalinecarboxylic acid (referred to hereafter as 'the quinoxaline compound') which they found on hydrolysis with NaOH. Accordingly, it was decided to find out whether paper chromatography could be used to detect these compounds. This work is described in the second part of this paper.

II. EXPERIMENTAL

(*a*) *Reagents*

(i) *Buffer Solutions.*—The following solutions were prepared from A.R. reagents and glass-distilled water:

A mixed acid solution 0.04M with respect to phosphoric, boric, and acetic acids.
Citric acid, 0.1M.
Boric acid, 0.2M.
Disodium hydrogen phosphate, 0.2M.
Glycine, 0.1M.
Sodium acetate, 1M.
Sodium borate, 0.05M.
Sodium diethyl barbiturate (veronal, B.P.), 0.1M.
Sodium hydroxide, 0.2M.
Hydrochloric acid.

Analysis with the dithizone method has shown heavy metals to be present in the test solutions in quantities less than 0.5 p.p.m. (total) and usually of the order of 0.1 p.p.m.

(ii) *Riboflavin Stock Solution.*—Riboflavin (25 mg) was dissolved in glass-distilled water, acidified with a few drops of glacial acetic acid, and made up to 500 ml; 1 ml of this solution contained 50 μg of riboflavin.

(*b*) *Method*

Riboflavin was assayed fluorimetrically with a Coleman Model 12B Photofluorimeter according to the method of Scott *et al.* (1946), but without the acid-acetone extraction, which was not necessary with aqueous solutions.

To confirm that the fluorescent quinoxaline compound was unlikely to interfere with the fluorimetric determination of riboflavin, a solution of the former of the same concentration as the riboflavin solutions used (5 μg/ml) was examined in the fluorimeter. The solution scarcely fluoresced in unfiltered ultraviolet light, and with the same filters as are used for riboflavin estimations there was no galvanometer deflection.

TABLE 1

LOSS OF RIBOFLAVIN FROM SOLUTION AT LOW pH

Reagent	pH	Percentage Riboflavin Recovered			
		After Time at 100°C		After 1 Hr Aeration	After 1 Hr 100°C, Then 1 Hr Aeration
		0 Min	60 Min		
Conc. H_2SO_4, 10 ml	0·73	96·2	98·8	98·8	—
Conc. H_2SO_4, 15 ml	0·30	88·5	90·6	98·5	100·0
75% H_3PO_4, 10 ml	1·38	94·2	94·2	98·8	—
75% H_3PO_4, 15 ml	0·78	91·1	90·0	98·5	100·0
Conc. HCl, 15 ml	0·75	88·7	89·7	95·3	98·2
Conc. HCl, 17·5 ml	0·60	83·5	83·5	95·0	100·0
1M NaAc:1M HCl buffer	1·02	93·5	91·2	98·6	98·5
1M NaAc:1M HCl, buffer	1·07	91·8	92·1	97·2	100·0
McIlvaine buffer	7·14	92·0	56·5	—	58·2
McIlvaine buffer	8·23	92·0	17·7	—	20·8

(c) *Procedure*

(i) *Riboflavin Recovery Tests*

pH 0-2.0.—Ten ml of riboflavin stock solution were diluted with water, the pH adjusted to the desired value with HCl, and the volume then made up to 100 ml.

pH 2.0-8.3.—Ten ml of riboflavin stock solution and 20 ml of McIlvaine's phosphate-citric acid buffer solution (Britten 1932) at the desired pH were mixed together and diluted to 100 ml.

pH values above 8.5.—Ten ml of riboflavin stock solution were diluted with water, the pH adjusted to the desired value with NaOH, and the volume then made up to 100 ml.

Each of these solutions contained 5 μg of riboflavin per ml. They were all treated similarly.

Twenty ml of the solution were sealed into 50-ml glass ampoules for tests at 120 and 80°C. These were wrapped in tin foil to protect the solution from light, and held in an oil bath at 120-3 and 79-82°C respectively. After 1 hr, the ampoules were cooled, opened, and riboflavin determined. For tests at 100°, the solution as prepared was boiled under reflux in a flask protected from the light. After 1 hr the cooled sample was assayed.

The pH of all test solutions was determined before and after heating. The values given are "mean" readings and all fell within a range of ± 0.1 pH units. All pH values reported were determined with the glass electrode.

(ii) *Experiments at Low pH*

One hundred ml of various solutions as shown in Table 1 were made up to contain 5 μg of riboflavin and the volume of acid or concentration of buffer shown in the table. pH and riboflavin concentration were determined immediately, and the sample divided into two parts. The first part was aerated for 1 hr at room temperature. The second was boiled for 1 hr, assayed, and in most cases then aerated for 1 hr at room temperature and again assayed.

(iii) *Determinations of Rate Constants*

Universal buffer solution.—Ten ml of riboflavin stock solution and 50 ml of the mixed acids buffer solution were mixed together and adjusted to the desired pH with 0.2M NaOH. The volume was then made up to 100 ml with glass-distilled water and the pH checked.

Phosphate-citric acid buffer solution (McIlvaine's).—Ten ml of riboflavin stock solution, x ml of 0.2M Na_2HPO_4, and $20 - x$ ml of 0.1M citric acid were mixed together in a 100-ml volumetric flask. The value of x depended on the pH desired. The solution was made up to volume with distilled water and the pH checked.

Sodium diethyl barbiturate-hydrochloric acid buffer solution.—These solutions were prepared as described above (see (ii)) using x ml of 0.1M sodium diethyl barbiturate solution and $10 - x$ ml of 0.1M HCl.

Glycine-sodium hydroxide buffer solution.—These solutions were prepared as described above, using x ml of 0.1M glycine and $10 - x$ ml of 0.1M NaOH.

All solutions were transferred to a 250-ml flask connected to a reflux condenser by means of a ground-glass joint, and heated within a small enclosed boiling water-bath fitted with a lid, so that no light fell on the solution. Samples were withdrawn at regular intervals, cooled, and 5-ml aliquots taken for assay.

(*d*) *Paper Chromatography*

Paper chromatography was used to investigate solutions of riboflavin which had been boiled under various conditions. All chromatograms were developed by ascending flow in closed tanks covered with brown paper to exclude light. Developing solutions were butanol-acetic acid-water (4:1:5) and butanol-pyridine-water (3:2:5). Whatman No. 3 MM chromatography paper was used throughout and the chromatograms examined in daylight and in ultraviolet light.

III. Results and Discussion

(*a*) *Recovery at Different Temperatures*

The first step was to repeat the work of Loy, Haggerty, and Combs (1951) in which solutions of riboflavin at various pH's and in various buffer solutions were held at 120°C. Temperatures of 100 and 80°C were also explored. As already noted, the concentration of riboflavin was only 5 μg/ml, not 50. The former is closer to potencies normally found in foods. Results are shown in Figure 1.

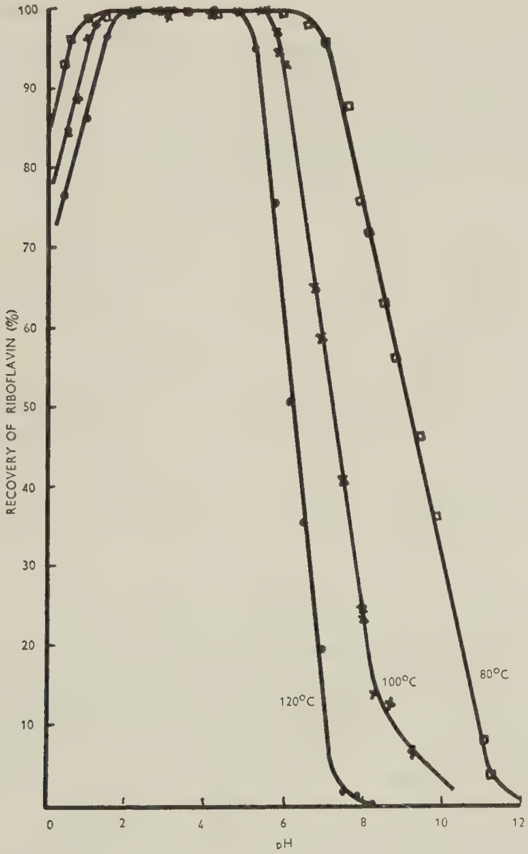

Fig. 1.—Recovery of riboflavin from solution (5 μg/ml) after heating at various pH for 1 hr.

It can be seen that the results obtained at 121-3°C are very close to those obtained by Loy, Haggerty, and Combs although the 'plateau' is rather narrower, the range of complete stability being from pH 2.0 to 5.0 (1.0-5.4 for Loy, Haggerty, and Combs). This is probably due to the much lower riboflavin concentration. The point of complete destruction (pH 8) is almost identical with that shown by the earlier workers.

As may be expected, the range of complete stability is lengthened by lowering the temperature. At 100°C it is 1.7-5.5 and at 80° 1.3-6.5.

It was then asked whether the loss of riboflavin at very low pH was a true thermal destruction, or whether it was in fact due to the high hydrogen ion concentration. Preliminary tests suggested the latter, as a loss obtained on making up the test solutions was not increased by subsequent boiling.

The series of experiments recorded in Table 1 was then carried out. It is seen that at low pH there is incomplete recovery of riboflavin from cold unheated solutions, and that subsequent boiling for 1 hr does not increase the apparent loss. On bubbling air through the solutions, either before or after heating, the loss can be almost completely recovered. This is in marked contrast to the behaviour of the solutions at alkaline pH, in which large losses occur and subsequent aeration has no effect. It would appear that the losses of riboflavin at very low pH are due to a reversible reduction very different from the irreversible hydrolysis which occurs at the higher pH.

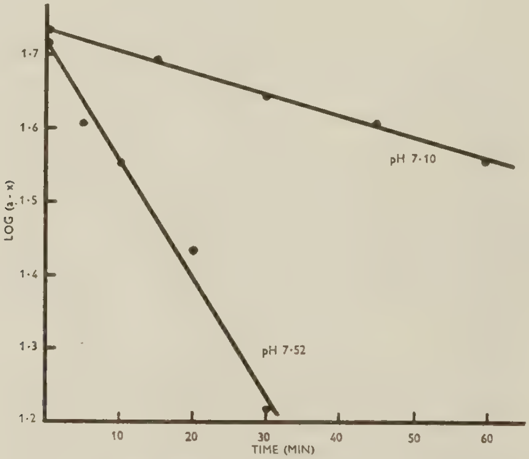

Fig. 2.—First-order reaction curves for riboflavin destruction at 100°C.

(*b*) *Effect of Buffer Anions*

Generally speaking, the results of this work have been interpreted in terms of simple reaction kinetics. The galvanometer reading for any given sample is proportional to the riboflavin in solution, and hence is equivalent to the term $(a-x)$ in the first-order reaction equation

$$k=\frac{1}{t}\ln\frac{a}{a-x}\ ,$$

where k is the rate constant, a the initial concentration of the substance studied, and x the amount of substance destroyed in t min.

That the thermal destruction of riboflavin is a first-order reaction is shown by the linearity of the curves in Figure 2 in which are plotted log $(a-x)$ against *t* for riboflavin destruction in, for example, phosphate-citric acid buffer solutions at pH 7.10 and 7.52. The temperature in the examples given was 100°C. Surrey and Nachod (1951) in their discussion of the alkaline hydrolysis of riboflavin showed from optical density figures that the degradation of a 5 per cent. solution in 0.25N NaOH at 25°C was a first-order reaction for which $k = 2.7 \times 10^{-4}$ min^{-1}.

From the gradients of these curves *k*, the rate constant, can be calculated, and this has been done for all buffer solutions studied. Log *k* has been plotted against pH and the curves shown in Figure 3 obtained.

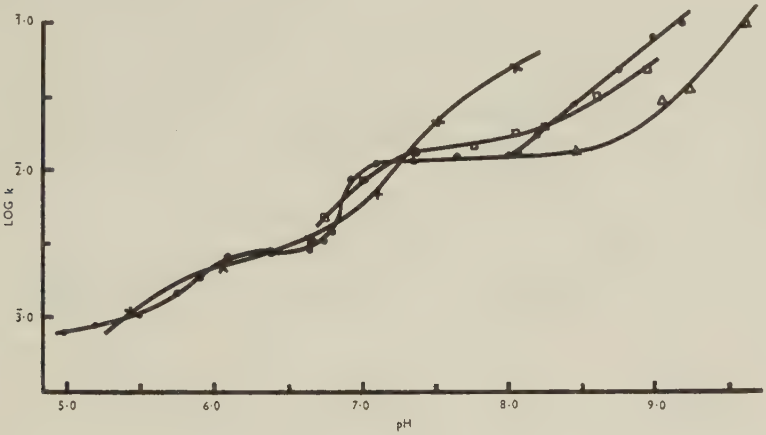

Fig. 3.—Log *k*/pH curves for the destruction of riboflavin in buffer solutions at 100°. ●, Modified universal buffer; X, McIlvaine's citric acid-phosphate buffer; ☐, veronal-HCl buffer; △, glycine-NaOH buffer.

The first buffer studied was the phosphate-citric acid (McIlvaine's) buffer. The shape of the curve strikingly resembles a similar curve obtained with aneurin in the same buffer solution, i.e. a sigmoid curve with points of inflexion corresponding with the points of inflexion in the titration curve of H_3PO_4 (Farrer 1945). With riboflavin this curve is displaced towards the higher pH values but nevertheless it would appear that the considerations of the effect of ionic environment on the rate of destruction of aneurin (Farrer 1945) apply also to riboflavin.

The pH/log *k* curve for the modified universal buffer solution shows the same effect and reinforces the suggestion that the destruction of riboflavin as well as that of aneurin proceeds at different rates under the influence of different anions. It is noteworthy that below pH 7, if pH alone and not buffer ions also were considered, the curve obtained would be almost linear. It is above neutral point that big differences appear. The modified universal buffer solution contains boric acid, which has been shown to form with riboflavin (Frost 1942) a complex which is stable on the alkaline side of 7 but is unstable on the acid side. This appeared to provide a likely explanation for the very different behaviour of riboflavin in the two buffer solutions at pH's above 7. However, of repeated attempts to demonstrate the presence of a complex by using paper chromatography, none succeeded. Frost (1942) has already failed to show that the complex had any effect on freezing point depression, electrometric titration, precipitation with heavy metals, specific rotation, and transmission curves.

When the barbiturate buffer was used, it was found that the curve obtained was very close to that of the universal buffer. This too discounted the suggestion that the shape of the latter was due to a boron-riboflavin complex.

Further point is given to the buffer effect by the results obtained with the glycine-NaOH buffer (pH range 8.2-10.0) It can be seen that it extends still further the range of pH maximum (of about 1.5 pH units) over which it is possible to obtain the same rate of destruction. These curves show clearly the effect of different anions at given pH values, especially above pH 7.

Calculations and some preliminary experiments suggest that the variations of ionic strengths in the concentrations of the buffer solutions employed will not be sufficient to influence the general conclusions to be drawn from Figure 3. The salt effect has not yet been studied.

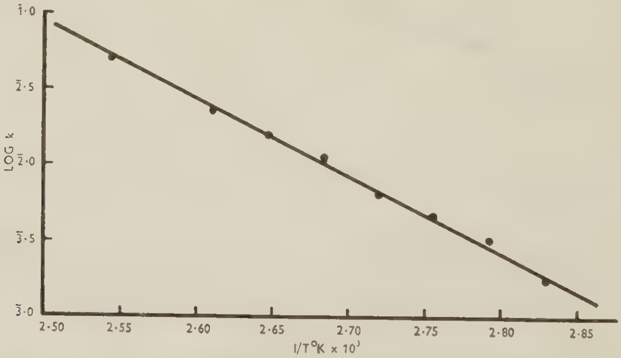

Fig. 4.—Arrhenius equation curve for riboflavin destruction in citric acid-phosphate buffer solutions at pH 7.27 ± 0.03.

(*c*) *Effect of Temperature*

Rate constant is related to temperature by the Arrhenius equation, which may be written as

$$\ln k = I - {}^{E}/_{RT},$$

where k is the rate constant, T the temperature (°K), and I, E, and R are constants. The curve log $k/{}^{1}/_{T}$ should thus be linear.

The thermal destruction of riboflavin was tested against this equation by determining k at eight temperatures from 80°C to 120°C in phosphate-citrate buffer solutions at pH 7.27. The curve obtained by plotting log k against ${}^{1}/_{T}$ is shown in Figure 4. It is apparent that the Arrhenius equation is followed very closely.

(*d*) *Effect of Concentration of Riboflavin*

To facilitate the chromatography of solutions of riboflavin heated in the presence of various buffers a concentration of 500 μg of riboflavin per ml of solution was used. It was noticed that spots of riboflavin persisted long after the time when, according to the thermal destruction curves for riboflavin solutions of 5 μg/ml, all the riboflavin should have been destroyed.

It seemed then that the rate of destruction of riboflavin depends also on its concentration in solution. This is not a new observation as Daglish, Baxter, and Wokes reported it in 1948 and Shimizu (1950) had also referred to it, but no attempt seems to have been made to express quantitatively what must be a simple relationship.

Accordingly the rate constant k was determined for riboflavin destruction at 100°C in universal buffer solutions at pH 7.70 ± 0.03 at concentrations of 5, 50, and 500 μg riboflavin per ml.

The results obtained were:

Riboflavin Concn.	k (min^{-1})
5 μg/ml	0.0155
50 μg/ml	0.0120
500 μg/ml	0.00823

If log concentration is plotted against k, a straight line results (Fig. 5). It has already been shown (Farrer 1948) that there is a relationship between rate constant and concentration of aneurin which underlines what is already clearly recognized, that the thermal breakdown of these substances is not a true first-order reaction.

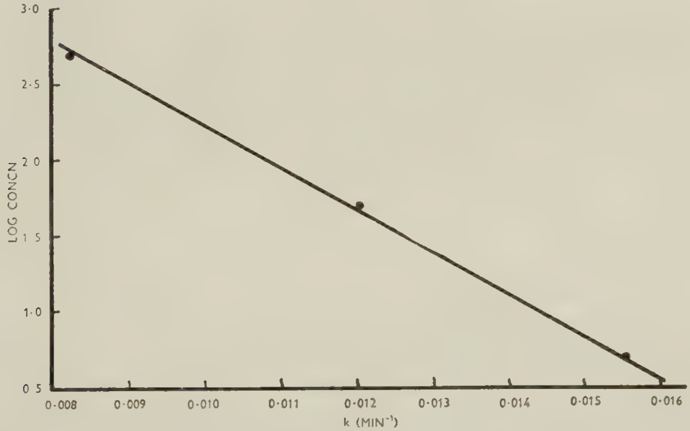

Fig. 5.—Relation between concentration of solution (μg/ml) and reaction velocity for the thermal destruction of riboflavin at 100°C in universal buffer solutions at pH 7.70 ± 0.03.

(*e*). *Thermal Degradation Products of Riboflavin*

The quinoxaline compound was prepared by the method described by Surrey and Nachod (1951), and the free acid and sodium salt chromatographed using butanol-acetic acid-water (4:1:5). Each gave a yellow spot which, when examined under ultraviolet light, emitted a characteristic aquamarine fluorescence. The R_F values of free acid and sodium salt were the same, 0.41.

To confirm that the quinoxaline compound could be detected in alkaline hydrolysates containing unchanged riboflavin, 1 g of riboflavin was dissolved in 250 ml of 0.2N NaOH, and heated at 100°C. Samples withdrawn at 10-min intervals were chromatographed for 4 hr at 20°C using butanol-acetic acid-water (4:1:5). At 0 min, the intense yellow riboflavin spot was obtained, together with an unidentified pale yellow spot seen in ultraviolet light only at the point of application of the solution. The latter has previously been reported by Hais and Pecakova (1949) and attributed by them to an adsorbate on the paper. In all samples taken at 100°C, exposure to ultraviolet light showed:

(i) The pale yellow spot, R_F value 0—an adsorbate on the paper;

(ii) A trace of an elongated bluish white spot, R_F value approximately 0.70, identified as lumichrome;

(iii) A spot consisting of an aquamarine fluorescence with a mauve upper edge suggesting a composite spot of two compounds. The R_F value was 0.49.

The best separation of the composite spot was obtained by developing the chromatogram with butanol-pyridine-water (3:2:5) and this solvent was used in all subsequent work unless otherwise stated. Comparative chromatograms run side by side then showed that the aquamarine spot in these mixtures was the quinoxaline spot, and that the mauve spot was due to a so far unidentified compound present apparently in very small amounts, and detectable also in the mother liquors remaining from the preparation of the quinoxaline compound according to Surrey and Nachod's directions. This substance is possibly a further breakdown product of the quinoxaline compound.

After 10 min at 80°C a similar solution, 1 g riboflavin in 250 ml 0.2N NaOH, showed only riboflavin and the adsorbate. Samples taken at 20, 30, and 40 min showed:

(i) The adsorbate,
(ii) Riboflavin,
(iii) The quinoxaline compound,
(iv) The unidentified mauve spot, and
(v) A trace of lumichrome.

Chromatograms were then prepared, as described above, from the solutions of riboflavin in various buffers used for the kinetic studies. These solutions contained originally 5 μg riboflavin per ml and proved to be too dilute to give detectable spots after hydrolysis.

More concentrated solutions (500 μg/ml) were therefore used for chromatographic studies. These follow the same kinetic pattern as the more dilute riboflavin solutions although the rate of reaction is slow, as has already been described.

Figure 6 is a typical chromatogram. It refers to a solution of riboflavin (500 μg/ml) at pH 8.12 in universal buffer solution which was heated at 100°C away from the light. Samples were withdrawn at 20-min intervals from 0 to 180 min, cooled, and spotted in duplicate. After development with butanol-pyridine-water (3:2:5) and drying, the chromatogram was examined in ultraviolet light and the spots observed were:

A, bluish white, elongated spot av. R_F 0.71. Lumichrome.
B, mauve av. R_F 0.63. Unidentified.
C, intense yellow av. R_F 0.53. Riboflavin.
D, aquamarine av. R_F 0.48. Quinoxaline compound.

In ordinary light, both *C* (riboflavin) and *D* (quinoxaline compound) are yellow.

Because of its low sensitivity (50 μg/ml was the smallest concentration which could be detected), the quinoxaline compound was not observed in this chromatogram until after 40 min hydrolysis. At this stage 55 per cent. of the riboflavin remained. After the appearance of the quinoxaline compound the riboflavin spots were clearly less intense and decreased in size until, at 180 min, the spot was quite small and only faintly yellow. The mauve spot appears at 80 min and the lumichrome spot is just discernible at the beginning of the hydrolysis.

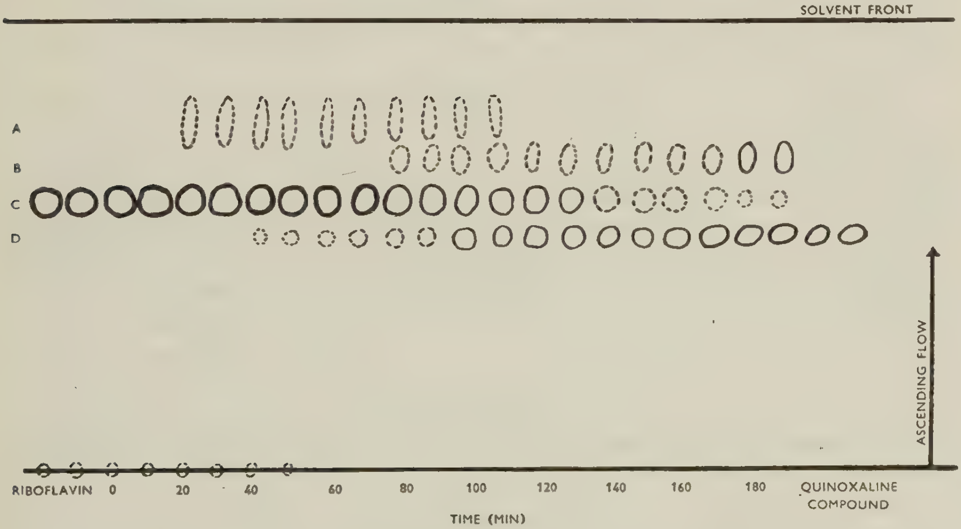

Fig. 6.—Chromatograms for hydrolysis of riboflavin at pH 8.12.

According to Surrey and Nachod (1951) urea too is found in alkaline hydrolysates of riboflavin. One would expect the degradation of urea to free ammonia under alkaline conditions, and this was detected with litmus paper when 1 g of riboflavin was heated for 30 min in 250 ml of 0.2N NaOH. A chromatogram of this solution was developed with the butanol-pyridine-water (3:2:5) solvent, and, when dry, sprayed with *p*-dimethyl-aminobenzaldehyde which gives a yellow spot with urea. Such a spot was found and compared with a urea chromatogram. The R_F value was 0.49.

Examination in ultraviolet light showed that the urea spot on the test chromatogram coincided with that of the quinoxaline compound, which is also yellow in ordinary light. Separation of the two was obtained on chromatograms developed with butanol-acetic acid-water (4:1:5). Thus, for example, urea and ammonia, as well as the quinoxaline compound, were obtained from a riboflavin solution (500 μg/ml) boiled for 3 hr at pH 7.80 in a McIlvaine citric acid-phosphate solution. None of them was obtained in a similar solution boiled at pH 6.13 for 4 hr in which no destruction of riboflavin would be expected because of the high concentration of riboflavin used.

These chromatograms are typical of all studied, and it is clear that the thermal degradation of riboflavin always followed the mechanism outlined by Surrey and Nachod (1951).

Shimizu (1951), in a second report only recently available in Australia, has also used paper chromatography for the study of the photodecomposition of riboflavin. He confirmed the occurrence of riboflavin and lumichrome, but noted that "the photodecomposition at higher temperatures in alkaline media seemed to belong to a completely different type of decomposition from those under other conditions." It would appear from our work that the decomposition under these conditions follows the course already described above, i.e. due to an alkaline hydrolysis, and is in no way the same as in irradiated solutions.

There still remained the question of the destruction at very low pH values, which seemed, at least in the greater part, to be very similar to the formation of leucoflavin.

Repeated chromatograms using butanol-pyridine-water (3:2:5) have shown only riboflavin in the solutions. Attempts have been made by using inert atmospheres to demonstrate the presence of leucoflavin, and even to chromatograph, for comparative purposes, chemically prepared solutions of leucoflavin. In every test only riboflavin was found. There is enough evidence, however, to permit us to say that the small destruction at very low pH is quite different in every way from that found at the higher pH values and from that already well described in the literature which occurs in irradiated solutions.

IV. Acknowledgment

The authors wish to thank the Directors of Kraft Foods Limited for permission to publish this paper.

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STUDIES ON THE ACTION OF RENNET ON CASEIN AND THE NATURE OF CLOTTING

By H. G. HIGGINS* and DALLAS FRASER*

[*Manuscript received June* 9, 1953]

Summary

Casein and paracasein preparations have been compared by various methods, including titration techniques, solubility, and ultraviolet spectra of the proteins and their azo-derivatives. It is suggested that rennet may release imidazole or phenolic groups from intramolecular linkage. The liberation of proteoses was confirmed but their presence does not appear to be essential for clotting. Support is given to the view that α-casein is more affected by rennet than are other casein components. The stability of the clots and the effect of electrolytes on the clotting process have been examined.

I. INTRODUCTION

The mechanism of milk clotting can be resolved into two stages: the conversion of casein into paracasein under the influence of the rennet, and the coagulation of the paracasein in the presence of calcium ions. Linderstrøm-Lang (1929), who first demonstrated the inhomogeneity of casein, advanced the hypothesis that rennet destroys, or inactivates, a "protective colloid" component, thus rendering unstable the calcium caseinates corresponding to the other components, and leading to precipitation or clotting. Alexander (1912) had previously put forward a somewhat similar hypothesis, in which lactalbumin was considered as the protective colloid. The work of Cherbuliez and Meyer (1933), Cherbuliez and Jeannerat (1939), and Cherbuliez and Baudet (1950) has indicated that α-casein may be regarded as the protective colloid in Linderstrøm-Lang's theory and that the calcium salts of β- and γ-casein, which are themselves insoluble, are kept in solution by the soluble calcium salt of α-casein, with which they are associated. Nitschmann and Lehmann (1947) have provided further support for Linderstrøm-Lang's hypothesis by showing that calcium ions cannot differentiate between casein and paracasein particles in a mixed solution.

Hammarsten (1872) postulated that the action of rennet on casein resulted in an insoluble curd and a soluble whey-protein (usually known in the literature as "Hammarsten's proteose"). Cherbuliez and Baudet (1950) regarded their soluble δ-fraction as this proteose. Earlier Bosworth (1913) had claimed that the appearance of the proteose was not due to the action of the rennet and in view of some confusion in the literature clarification of the following points seemed desirable: whether the enzyme is really responsible for the liberation

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of the proteose, and, if this is so, whether the reaction or the proteose itself are essential for the clotting process.

The assumption sometimes made that the rupture of peptide bonds is the basic feature of the casein-paracasein transformation probably arises from the observation that various proteolytic enzymes can coagulate milk, but it has actually received little experimental support. Wright (1924) had concluded from the similarity of the racemization curves for casein and paracasein that rennet does not cause proteolytic cleavage of the protein, and Holter (1932) was able to obtain, without apparent increase in amino or carboxyl groups, paracasein which could be completely precipitated by calcium ions. More recently Nitschmann and Varin (1951) have concluded that clotting can take place with negligible proteolysis, amounting to the rupture of only one peptide link in 10,000. Berridge (1945) demonstrated that commercial rennet is a mixture of pepsin and rennin, and that rennin itself also exhibits considerable proteolytic activity. The relevant question therefore is not whether proteolysis of the casein occurs, but whether it is essential for coagulation. Nitschmann and Lehmann (1947) suggested that the essential chemical change in the transformation of casein into paracasein might not lie in a primary valence reaction, but that the rennet might act as a specific "denaturase" for one of the casein components.

Some clarification of these questions was sought in the experiments described below.

II. Materials and Methods

(*a*) *Protein Preparations*

Casein and α-casein for the experiment described in Section III(*e*) were prepared from the same batch of milk according to the methods of Warner (1944), except for minor deviations.

Casein and paracasein for comparative studies were prepared under strictly parallel conditions, except for the rennet treatment, as described by Higgins and Hayes (1951). Two pairs of preparations were used.

(*b*) *Acid Hydrolysates*

These were prepared by refluxing 0.5 g air-dry protein with 10 ml 6N hydrochloric acid for 20 hr.

(*c*) *Rennet*

A commercial preparation (New Zealand cheese rennet) was used.

(*d*) *Electrometric Titration*

The pH values were measured by means of a glass electrode system. The proteins (2.0 g) were dissolved in 40 ml N/20 sodium hydroxide and titrated with 1N hydrochloric acid. Acid groups were calculated from the equivalent between pH 6 and pH 2. Controls were run in the absence of protein and appropriate corrections applied.

(*e*) *Formol Titration*

The method of Kekwick and Cannan (1936) was adopted. The formaldehyde was neutralized, the protein solutions (1 g in 50 ml N/50 sodium hydroxide) were adjusted to pH 9, and were titrated back to this point after the addition of the formaldehyde.

(*f*) *Titration in Organic Solvents*

The methods of Linderstrøm-Lang and Jacobsen (1940) were applied, the titrations in ethanol being carried out with N/10 sodium hydroxide in 90 per cent. ethanol to the thymolphthalein end-point (normally pH 9.3-10.5), and those in acetone with N/10 hydrochloric acid in 90 per cent. ethanol to the naphthyl red end-point (normally pH 3.7-5.0). The original solutions were made up by dissolving 0.2 g protein, with agitation, in 20 ml N/20 sodium hydroxide and adjusting to pH 6, following Lea and Hannan (1950), with 1N hydrochloric acid (*c.* 0.8 ml). Titrations were carried out after the addition of (i) 15 ml ethanol plus 2.5 ml thymolphthalein (0.5 per cent.), (ii) 50 ml ethanol plus 2 ml thymolphthalein, (iii) 10 ml acetone plus 0.5 ml naphthyl red (0.01 per cent.), (iv) 30 ml acetone plus 1 ml naphthyl red. Controls were run on volumes of water equal to those of the protein solutions, adjusted to pH 6 and with the solvent and indicator added.

(*g*) *Total Nitrogen*

The Kjeldahl method was used, with parallel duplicates of each preparation.

(*h*) *Amino Nitrogen*

Determinations were made by the Van Slyke nitrous acid procedure in the manometric apparatus (Peters and Van Slyke 1932).

(*i*) *Optical Absorption*

Extinctions and absorption spectra were measured with a Unicam S.P. 500 spectrophotometer, using 1-cm cells and water as the reference medium. No transverse measurements of the scattered radiation were made, but high optical clarity was assumed in dilute protein solutions which had been filtered through sintered glass.

(*j*) *Solubility*

The proteins (0.05 g) were equilibrated at 25°C with 5 ml solvent and centrifuged to remove the undissolved protein. Extinctions were measured at 277 mμ on the supernatants.

(*k*) *Coupling with Diazotized Sulphanilic Acid*

Coupling was carried out as described previously (Higgins and Fraser 1952). The proteins (0.01 g) were first dissolved in *c.* 5 ml N/20 sodium hydroxide for 5 min and the solutions acidified with 1N hydrochloric acid.

III. Procedure and Results

(*a*) *Proteolysis. Acid and Basic Groups*

The various titration techniques were used to determine the state of the acid and basic groups in casein and paracasein preparations. As these procedures involved dissolving the protein in sodium hydroxide, an examination was made of the rate of hydrolysis of casein under the conditions used. The rate of rupture of peptide bonds was found, by titrations in alcoholic sodium hydroxide, to be about 2 per hour per 10^5 g casein in the initial stages, which is not great enough to introduce serious errors during the periods used to dissolve the casein (usually about ½ hr).

The pH values obtained upon dissolving 1.0 g protein in 50 ml N/50 sodium hydroxide were, for paracasein: 8.34, 8.32, and for casein: 9.55, 9.40. After adjustment to pH 9 and addition of formaldehyde for the formol titration the pH values were respectively 6.47 and 6.44.

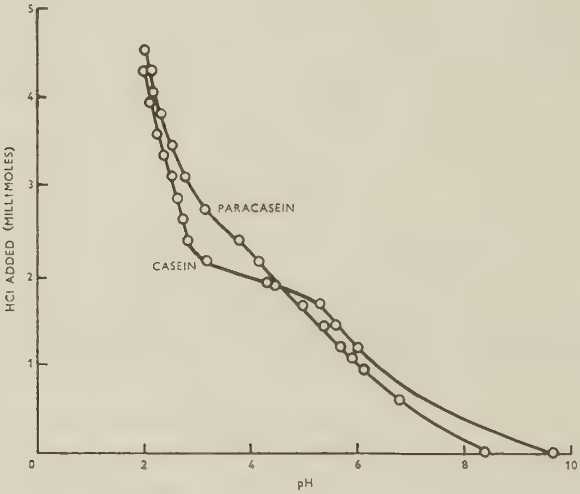

Fig. 1.—Electrometric titration of casein and paracasein. Amount of 1N HCl added to 2.0 g protein dissolved in 40 ml 0.05N NaOH.

Typical electrometric titration curves for casein and paracasein are shown in Figure 1. The titration equivalent between pH 2 and pH 6 has been used as a measure of free carboxyl groups by, for example, Herriott (1947), who drew attention to the uncertainty arising from the overlapping of the carboxyl and histidine titration regions. Although the method has been used for casein by Lea and Hannan (1950), a further objection arises from the presence of phosphoric acid groups. These have been taken into account by Hipp, Groves, and McMeekin (1952) on the assumption that the phosphoric acid affects the titration curve of casein as it does that of egg albumin (Cannan, Kibrick, and Palmer 1941). The total acid groups titratable between pH 2 and pH 6 have been calculated and are given in Table 1. The data have been corrected for the controls, but as these are the same for each preparation, the magnitude of the increase upon rennet treatment is not affected.

TABLE 1

COMPARISON OF CASEIN AND PARACASEIN FROM TITRATION DATA

Titration (see text)	No. of Groups per 10^5 g Air-dried Protein			
	Casein	Paracasein	Increase	
Electrometric	131	152	21	
Formol	73	69	−4	
Base in ethanol (15 ml)	58·6	80·1	21·5	Mean 21
Base in ethanol (50 ml)	90·4	110·0	19·6	
Acid in acetone (10 ml)	77·6	92·4	14·8	Mean 15
Acid in acetone (30 ml)	88·8	103·0	14·2	

The ε-amino groups of lysine were calculated from the formol titration equivalents. The apparent small decrease upon rennet treatment (Table 1) may be due to the difference in ionic strength consequent upon adjusting to pH 9; Kekwick and Cannan (1936) drew attention to the large effect of salt upon the ionization of the protein.

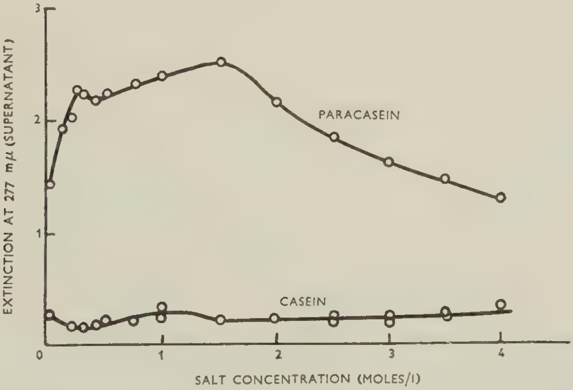

Fig. 2.—Solubility of casein and paracasein in the absence of calcium.

The interpretation of the ethanol and acetone titration data (Table 1) varies according to whether or not preliminary neutralization of the protein solution is effected (Richardson 1934), and there is also some disagreement between various authors (Richardson 1934; Linderstrøm-Lang and Jacobsen 1940; Lea and Hannan 1950).

The liberation of proteose by rennet was confirmed experimentally by examination, against appropriate controls, of the supernatants from the clotted sodium caseinate solutions of concentration 3.1 per cent. and pH 6.7, after adjustment to pH 4.2-4.3. The tests, which are described in detail in an unpublished report by the authors, showed that the material liberated from the protein by rennet action was inhomogeneous and could be fractionated by conventional methods. It was observed that paracasein after removal of the proteose-containing supernatant and re-dissolving (3 per cent. solution) could still be clotted with calcium chloride.

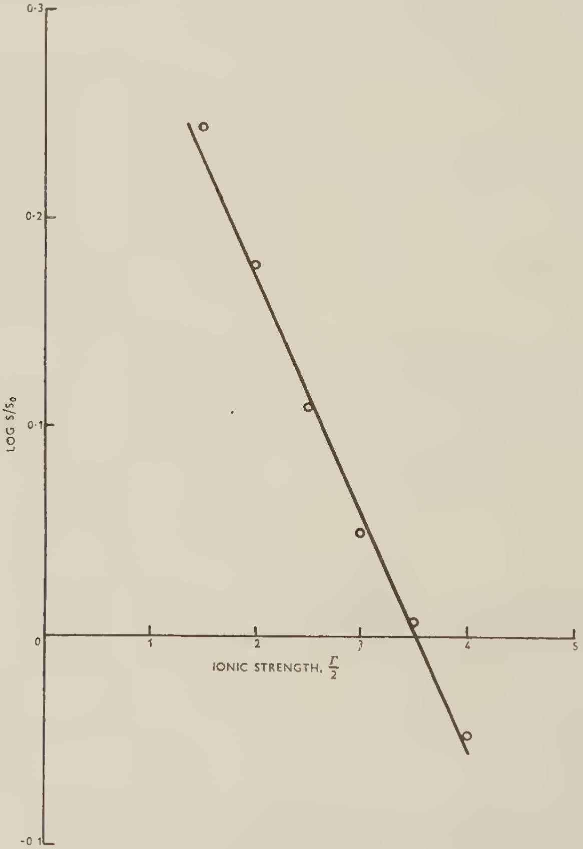

Fig. 3.—Salting-out of paracasein in ammonium chloride.

(*b*) *Solubility of Casein and Paracasein*

The solubility of casein in solutions of neutral salts is very considerably increased upon treatment with rennet, as shown in Figure 2 for ammonium chloride solutions. Apart from its greater solubility, paracasein exhibits very much greater sensitivity towards the concentration of salt. The subsidiary maximum at *c.* 0.25 mol/l suggests the presence of a component or group of components which can be salted out at concentrations much lower than that at which the main component reaches its maximum solubility (*c.* 1.5 mol/l). In Figure 3 the salting-out characteristics of paracasein are shown above this concentration. It is not even possible to derive such a curve from the data for casein for which log S/S_0 is practically zero. From the slope of the line in Figure 3 the salting-out constant for paracasein can be calculated (Cohn and Edsall 1943) as 0.114.

Despite its greater solubility in salt solutions at protein concentrations not greatly in excess of the solubility, paracasein shows a lower solubility at high protein concentrations. Thus when 16 g protein were equilibrated with 100 ml M/15 phosphate buffer of pH 6.9 by vigorous shaking at 25°C, the apparent solubilities were:

Casein 11.1 g/100 ml;
Paracasein 3.6 g/100 ml.

Table 2

ULTRAVIOLET ABSORPTION OF CASEIN AND PARACASEIN

Protein	Solvent	Extinction (1 g/l)	
		277, 280 mμ	320 mμ
Casein (12·15% N*)	M/15 phosphate buffer pH 6·8 M/20 borax buffer pH 9·2 N/10 sodium hydroxide	1·02 (277) 0·98 (277) 1·07 (280)	0·231 0·222 0·214
Paracasein (12·23% N*)	M/15 phosphate buffer pH 6·8 M/20 borax buffer pH 9·2 N/10 sodium hydroxide	1·06 (277) 1·03 (277) 1·10 (280)	0·240 0·230 0·222

* On an air-dry basis. Correction for moisture would increase these figures considerably.

(*c*) *Ultraviolet Absorption of Casein and Paracasein*

Porcher, Volkringer, and Brigando (1934) demonstrated the similarity of the ultraviolet absorption spectra of casein and paracasein. Spectra observed on one pair of the present preparations in phosphate buffer of pH 6.9 showed a maximum at *c.* 277 mμ, but the extinction coefficient at this wavelength was nearly 4 per cent. higher for paracasein after correcting for haze by subtraction of the extinction at 320 mμ (Beaven and Holiday 1952). This effect was examined, in relation to total nitrogen and at different pH values, with casein and paracasein derived from another batch of milk. The extinctions for paracasein corrected for haze and for nitrogen were higher in each case than those for casein by amounts in the vicinity of 3 per cent., as shown in Table 2.

Both casein and paracasein solutions were found to conform closely to Beer's Law at 280 mμ up to concentrations of at least 1 g/l when dissolved in phosphate buffer of pH 6.8.

(*d*) *Reaction of Casein and Paracasein with Diazonium Compounds*

The results of previous studies on the reaction of amino acids with diazonium compounds have been applied to the problem of the casein-paracasein transformation. The spectra shown in Figures 4 and 5 refer to different series of intact casein and paracasein preparations, made from two batches of milk. Similar experiments were made with acid hydrolysates prepared from the same protein preparations used in obtaining the spectra shown in Figure 4. The hydrolysates were first neutralized with sodium hydroxide, and ethanol was omitted from the coupling media because it increases the instability of azo-amino derivatives. The spectra obtained are shown in Figure 6 for a concentration at which all the potentially reactive groups in the hydrolysates can react with the diazonium salt. The data of Figures 4, 5, and 6 have been converted to a protein concentration of 1 g/l, but no correction has been made for other absorbing components, such as excess reactants. The only appreciable error thus introduced will result from the presence of unreacted *p*-diazobenzenesulphonic acid.

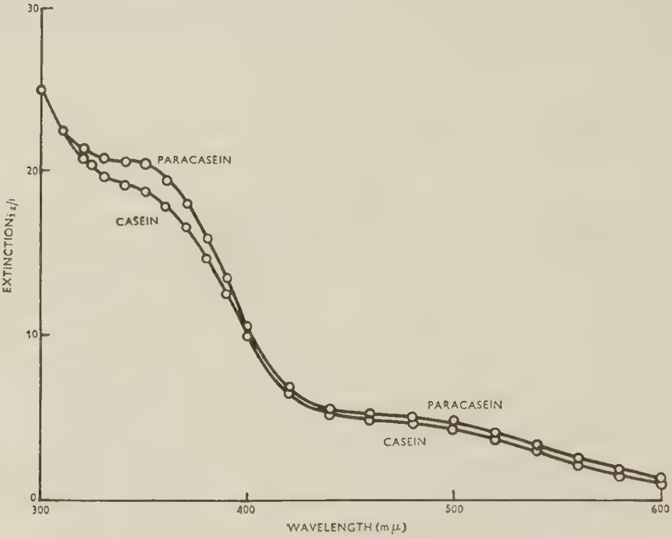

Fig. 4.—Absorption spectra of azo-derivatives of casein and paracasein (0.01 g/25 ml).

As the interpretation of these data involves the amino content of the proteins and their hydrolysates, the amino groups were determined, with the following results (expressed as equivalents $NH_2/10^5$ g air-dry protein): intact casein 43.7; intact paracasein 47.0; casein hydrolysate 665; paracasein hydrolysate 690.

(*e*) *Clotting of α-Casein*

The following experiment was carried out to determine the relative responses of whole casein and α-casein towards rennet treatment. To 5-ml aliquots of 1 per cent. solutions of each (on an air-dry basis), adjusted to pH 6.64, were added 0.05 ml rennet solution and 0.2 ml 1M calcium chloride solution. Clots formed after *c*. 10 min at 25°C. The supernatants were filtered off through sintered glass and their extinctions measured against water at 277 mμ. They were:

Whole casein 1.99;
α-Casein 1.52.

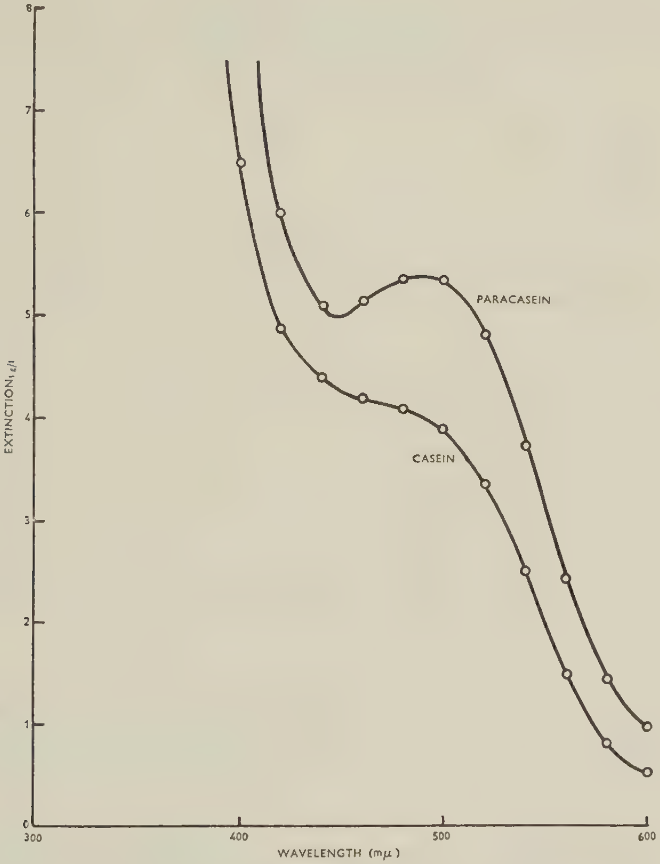

Fig. 5.—Absorption spectra of azo-derivatives of casein and paracasein (0.01 g/25 ml).

(*f*) *Effect of Ionic Strength and Anion Valency on Rate of Clotting*

Experiments based on the investigations of Lovelock and Porterfield (1952) on the clotting of blood plasma were carried out with paracasein. The solutions, of final concentration 3 g/100 ml adjusted to pH 6.4 with sodium hydroxide, were maintained at 25°C under the various conditions shown in Table 3, which indicates whether or not a clot formed 30 min after the addition of the calcium chloride. It was observed that, where clots formed in the presence of potassium ferricyanide, the process was almost instantaneous and they were more rigid and coherent than those formed in the presence of electrolytes of lower anion valency.

(*g*) *Stability of the Clots*

In order to obtain further information on the nature of the intermolecular bonds within the rennet-sponsored clots, the stability of the clots was examined under various conditions. To three 10-ml aliquots of a 3 per cent. solution of paracasein of pH 6.7, 1 ml 1M calcium chloride was added, the clots were allowed to form, and the supernatants were removed. Five ml of the reagents shown in Table 4 were added, one to each clot, with the results indicated.

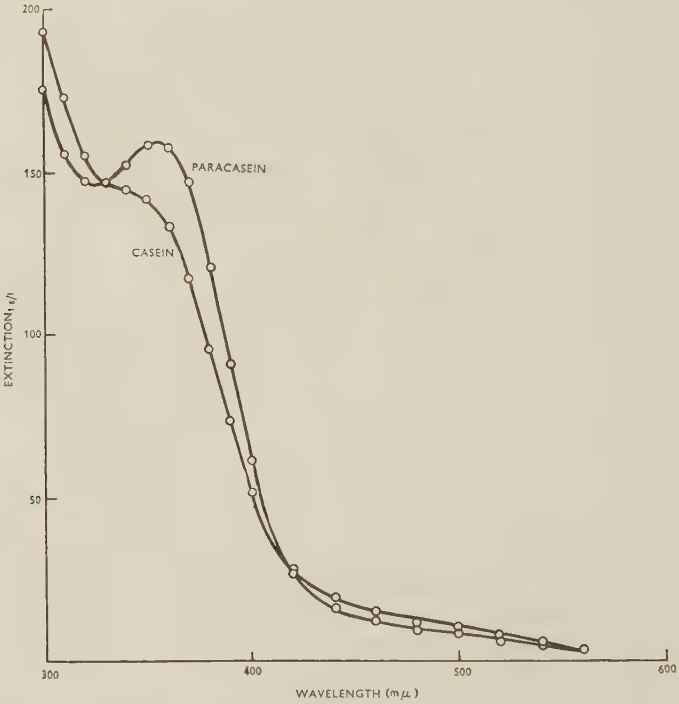

Fig. 6.—Absorption spectra of acid hydrolysates of casein and paracasein, after reaction with diazotized sulphanilic acid (0.01 g intact protein/25 ml).

IV. Discussion

The interpretation of the titration data is by no means unequivocal, but they assist in evaluating the change brought about in the casein upon rennet treatment. The electrometric titration curve shown in Figure 1 shows the greater binding capacity of the paracasein above pH 6.8 (also shown by its lower equilibrium pH value in the presence of a given quantity of sodium hydroxide) and between pH 5.2 and pH 3.2.

If the ethanol and acetone titrations are interpreted according to Lea and Hannan (1950), who were working with casein, it can be concluded from Table 2 that the increase in basic groups upon rennet treatment is greater by six than that in the acid groups. This cannot be ascribed to α-amino (since the appearance of each group of this type would be accompanied by a corresponding carboxyl), to ε-amino (from the formol titration), or to guanidino (since arginine is not determined) and so attention can be directed to the imidazole group of histidine. Richardson (1934) maintained that the scission of ester bonds will be estimated by the alkaline titration but not by the acid, and this could also account for the observed differences if his interpretation is correct.

TABLE 3

EFFECT OF IONIC STRENGTH AND ANION VALENCY ON CALCIUM CHLORIDE CONCENTRATION REQUIRED FOR CLOTTING

Crosses indicate clotting; dashes no clotting

Electrolyte		Sodium Chloride				Potassium Ferricyanide			
Molarity		0·1	0·2	0·3	0·4	0·1	0·2	0·3	0·4
Ionic Strength*		0·1	0·2	0·3	0·4	0·6	1·2	1·8	2·4
Final molarity of calcium chloride	0·018	+	—	—	—				
	0·045	+	—	—	—				
	0·072	+	+	+	—				
	0·090	+	+	+	—	—	—		
	0·18					—	—	—	—
	0·27					—	—	—	—
	0·38					—			+
	0·45	+	+	+	+	+	+	+	+
	0·91					+	+	+	+

* Taking molarity as molality and assuming complete ionization.

TABLE 4

STABILITY OF CLOTS

Reagent	Result
1N NaOH	Clot dissolved rapidly
1N HCl	Clot did not dissolve
Saturated NH_4Cl	Clot dissolved slowly

The observation that paracasein, after removal of the proteose-containing supernatant and re-solution, could still be coagulated suggests that the liberated proteose is not essential for clotting, a view also favoured by Cherbuliez and Baudet (1950), although the possibility remains that a substance in the proteose is strongly adsorbed on the paracasein. The possibility that liberation of the proteose exposes functional groups which can then react with calcium ions is also not precluded.

The relatively low solubility of paracasein at high protein concentration may be due to salting out by a small component or to complex formation between components of the protein mixture.

The differences in the ultraviolet absorption of casein and paracasein suggest that rennet may affect the absorbing capacity of the tyrosine groups, perhaps by rupturing intermolecular hydrogen bond structures involving the phenolic hydroxyl, and a detailed spectrophotometric study of this question might well be warranted. Paracasein exhibits greater coupling capacity with diazonium salts than casein, as shown by the higher extinctions of the azo-derivatives (Figs. 4 and 5). The absorption in the region 480-500 mμ can be related to the imidazole or phenolic hydroxyl groups (Higgins and Fraser 1952) and that at *c.* 360 mμ to amino groups (from unpublished data of the authors). The spectra of the azo-derivatives of the hydrolysates (Fig. 6) do not reveal a higher histidine or tyrosine content in the paracasein, as shown by extinctions at 480-500 mμ and at 325 mμ, and differences in the intact protein thus appear to be related to the accessibility of the groups concerned.

The suggestion that rennet may release some of the imidazole rings from intramolecular linkages is in accordance with the indirect indications of the ethanol and acetone titrations. D'yachenko (1950) also refers to the release of the imidazole ring during rennet fermentation, on the basis of potentiometric titration data, although he does not assign to this reaction a subsequent role in the clotting process.

According to the definition of denaturation given by Neurath *et al.* (1944), paracasein may perhaps be considered as a denatured protein in that the reactivity of certain side chains is apparently increased by intramolecular modification. On the other hand, the striking increase in solubility (in the absence of calcium ions) and the maintenance of molecular symmetry, as determined by viscosity measurements (D'yachenko 1950; Higgins and Hayes 1951), upon rennet treatment of casein are changes in the opposite sense to those usually encountered in denaturation processes. Like casein, paracasein also yields negative sulphydryl tests.

The data on the clotting of α-casein and whole casein suggest that the α-casein enters more fully into the structure of the clot, which supports the idea that the α-casein is more affected by the rennet than the other components. This result is in accordance with those of Pyne (1951), who found that, after rennet treatment, whole casein required a higher calcium ion concentration than α-casein to effect the same rate of coagulation.

The variation with ionic strength of the amount of calcium required for coagulation in the presence of sodium chloride shows that a certain electrolyte balance is necessary for clotting, as for blood plasma (Lovelock and Porterfield 1952). Whether, as these authors consider is the case for plasma, the function of the calcium is to maintain the surface charge of one or more of the colloidal components at a value suitable for interaction with the others, or whether D'yachenko and Nitschmann and Lehmann are correct in regarding the calcium ions as acting as bridges between paracaseinate molecules, is still open to doubt.

Although potassium ferricyanide requires a higher calcium ion concentration for clotting than sodium chloride, on a molar basis, the clots form almost instantaneously when the electrolyte balance is suitable and are more rigid and coherent. A possible explanation of this behaviour is that the trivalent anion facilitates three-dimensional cross-linking through calcium bridges attached to acid groups in different protein molecules.

The observations on the stability of the clots, considered in conjunction with the data discussed above, seem to be compatible with the formation of intermolecular bonds of a predominantly ionic character.

V. Acknowledgments

Experimental assistance was received from Mr. K. J. Harrington. The continued interest of Mr. G. Loftus Hills has been greatly appreciated, as have discussions with Mr. J. W. Legge and Mr. K. F. Plomley.

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THE AMINO ACID COMPOSITION OF KERATINS

I. THE AMINO ACID ANALYSIS OF MERINO 64'S QUALITY VIRGIN WOOL

By D. H. SIMMONDS*

[*Manuscript received October* 13, 1953]

Summary

The ion exchange chromatographic technique of Moore and Stein (1951) has been used to estimate the amino acids in an acid hydrolysate of merino 64's quality virgin wool. A complete analysis of one sample of this wool is summarized in Table 3 together with figures for the carbon, hydrogen, nitrogen, sulphur, and ash contents. With the exception of the glycine analyses the results confirm the figures previously accepted as reliable for wool.

I. INTRODUCTION

Although many estimations of individual amino acids in wool keratin have been made, no complete analysis of a single wool sample has yet been reported. Martin and Synge (1941*a*, 1941*b*, 1941*c*) and Gordon, Martin, and Synge (1943) were the first to evolve a method in which most of the neutral amino acids could be determined in the same sample of protein hydrolysate. This was achieved by counter-current extraction and by partition chromatography of the N-acetyl-amino acid derivatives. Previously, except for the work of Abderhalden and Voitinovici (1907), who used the ester distillation procedure of Fischer (1901), methods specific for individual amino acids had been used. The results of these early investigations are summarized in Table 1. For purposes of comparison all figures have been recalculated to amino acid nitrogen as percentage of total nitrogen, 16.5 per cent. being assumed to be the percentage total nitrogen in dry wool where no other data were given.

The present communication reports the complete amino acid analysis of a sample of 64's quality merino wool, as determined by the ion-exchange chromatographic method of Moore and Stein (1951), in conjunction with specific methods for cystine, methionine, tryptophan, and proline.

II. EXPERIMENTAL AND RESULTS

(*a*) *Preparation of Wool for Analysis*

Samples of 64's quality virgin merino wool (5 g) were Soxhlet-extracted for 5 hr with 150 ml of 95 per cent. ethanol. The wool was squeezed, dried, and extracted with 200-ml portions of distilled water, until no further material absorbing light of wavelength 270-290 mμ was leached out. The wool was then air-dried, hand-picked to remove traces of seed and other impurities, and stored until required.

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For the analyses, 3.0-g samples of extracted, air-dried wool from which discoloured tips had been removed were dried *in vacuo* over phosphorus pentoxide to constant weight. From these, three 0.05-g samples were weighed out and dried to constant weight in an oven at 110°C. Nitrogen was determined in the oven-dried samples by the micro-Kjeldahl technique. At the same time, 0.500 g (corrected for moisture content as determined above) was refluxed with 100 ml 6N HCl (twice glass-distilled) on an oil bath at 140-150°C for 16 hr. The HCl was removed by vacuum distillation and the concentrated solution was quantitatively transferred to a 50-ml measuring flask and made up to volume. For each analysis, 0.5 or 1 ml of this solution, as required, was measured into a small polythene cup and placed in an evacuated desiccator over P_2O_5 and KOH to remove water and HCl. Micro-Kjeldahl nitrogen determinations on the hydrolysate solution showed that manipulative losses were negligible.

(*b*) *Preparation of Columns*

Finely ground Dowex 50 was wet-sieved through a 250-mesh sieve and treated as described by Moore and Stein (1951). The chromatographic columns were constructed from coarse porosity sintered glass funnels (SF8A, porosity 1 or 2) sealed to "Pyrex" tubing of 0.8-0.9 cm internal diameter drawn down to a rounded point as close to the sintered disc as possible. A B14 standard taper joint was sealed to the top of the column, which was surrounded by a jacket through which water could be circulated. The columns were graduated at 100 cm above the sintered glass plate for the separation of the acidic and neutral amino acids, and at 15 cm for the separation of the basic amino acids. A slurry of Dowex 50 in 0.2N NaOH was poured into the columns until, on settling under air pressure equivalent to 15 cm of mercury, the tube was filled with resin up to the graduation. To obtain columns having a uniform particle size gradient from top to bottom and giving amino acid bands of satisfactory sharpness, the resin bed was backwashed with 0.2N NaOH and allowed to settle with gentle tapping by hand or by rubber pressure tubing attached to the shaft of an electric motor. Columns were checked by means of a spirit level to make sure they were vertical before settling. Final adjustment to the graduation mark was made by pressurizing the column as required. When the 0.2N NaOH level had fallen to within 0.25 cm of the top of the resin, the appropriate buffer solution was carefully applied without disturbing the surface. The 100-cm columns were treated with 150-200 ml of pH 3.29 ± 0.01 citrate buffer, and the 15-cm columns with 50 ml of pH 5.00 ± 0.01 citrate buffer before use.

To regenerate the columns after a run they were treated with 0.2N NaOH until the effluent was alkaline. They were then backwashed, allowed to settle, and equilibrated with buffer as described above.

(*c*) *Preparation of Buffer Solutions*

Except for omission of the detergent, the buffer solutions were prepared as described by Moore and Stein (1951) and stored at 5°C with the addition of thymol as a preservative. Just before use the appropriate quantity of buffer

Table 1

THE AMINO ACID COMPOSITION OF WOOL

Reference* / Amino Acid	1	2, 3	4	5, 6	7	8, 9, 10	11	12	13, 14, 15	16	17	Miscellaneous Results
Alanine	4·2						3·9		3·2			
Arginine		18·4	15·2		18·2			16·6	20·0	21·1	19·6	20·2 (18), 12·3 (19), 19·9 (20), 19·4 (21)
Aspartic acid	1·5			4·6					3·8	4·7		3·73 (22)
Amide N		6·6		7·3		7·2					7·6	8·6 (23), 7·2 (24), 8·7 (25)
Cystine	5·1	8·5			9·9			8·5	8·0	9·9	5·6	8·0 (26), 6·1 (27), 7·4 (28), 9·0 (29), 8·4 (30), 6·9 (20), 8·3 (32), 8·3 (33), 8·9 (48), 8·4 (22), 7·9 (21), 9·2 (34)
Glutamic acid	7·4			8·7					7·0	9·2		9·3 (30)
Glycine	0·7				7·9							
Histidine		10·5	1·1		1·2					1·8	4·5	1·2 (19)
Hydroxylysine												0·11 (35), 0·11 (24)
Isoleucine						7·2				3·0		
}	7·4					7·4	6·2		6·7			
Leucine										5·3		
Lysine		3·02	2·7	3·8	3·1					3·9	7·7	2·5 (19), 3·8 (36)
Methionine						0·4	0·2			0·4		0·25-0·38 (37), 0·34 (38), 0·11 (39), 0·32 (21), 0·35 (48)
Phenylalanine					2·2	1·9	0·8		1·9	2·1		
Proline	3·2					4·9	5·2		3·8	6·1		
Serine	0·08					7·5		7·6	}12·8			8·2 (23), 7·7 (26)
Threonine						4·7				4·8		4·6 (23)
Tryptophan		1·4			0·6							
Tyrosine	1·4	2·1			2·3		2·8	2·8	2·6	2·7		2·4 (40), 2·7 (41), 2·7 (42)
Valine	2·0					3·4	3·9		3·6	4·2		
Total estimated	32·6	40·0†	19·0	24·4	45·4	37·2	23·0	35·5	73·4	79·2	44·9	
Total N		17·6–18·0	16·57		15·4			16·67	16·9	16·2	16·14	16·50-17·07 (43), 16·4 (30), 16·57 (24), 16·43 (19), 16·3 (48), 16·3 (23), 16·29 (32), 16·66 (33), 16·50 (28)
Total S		3·52–3·59	4·52		3·6			3·5	3·76		2·91	2·96-3·97 (37), 3·34 (32), 3·19 (33), 3·47 (28), 3·39-3·73 (44), 3·64-3·35 (45), 3·58 (40), 3·63 (48), 3·35 (39), 3·43 (34), 3·03 (46), 3·50 (26)
Mean residue weight	118 (31), 116, 107 (47)											

was boiled to expel air, placed in a separating funnel, and covered with a layer of paraffin oil. To achieve satisfactory separation of glycine, alanine, cystine, and valine, it was found necessary to adjust the pH of the citrate buffer to 3.29, instead of 3.42 as specified by Moore and Stein (1951). The optimum value for this buffer can only be determined by trial and error in successive runs.

The ninhydrin reagent was prepared according to the directions of Moore and Stein (1948) but the solution used for diluting the colour developed after heating was a 50 per cent. (v/v) ethanol-water mixture in place of the *n*-propanol mixture recommended by them. The ninhydrin reagent and the diluent were both dispensed by pipettes each consisting of a hypodermic syringe connected through a three-way "Pyrex" stopcock to a reservoir on one side and a constricted outlet on the other. One bore of the three-way tap was blocked to prevent liquid passing directly from the reservoir to the outlet. The pipettes were calibrated by discharging them the requisite number of times into a graduated flask. This procedure was followed each day to draw off reagent solution which had been standing in contact with the polyvinyl chloride tubing connecting the pipette to the reservoir, otherwise the first three or four tubes examined were slightly turbid.

(*d*) *Measurement of Colour Yields and Calibration of the Spectrophotometer*

The colour yields for seven of the amino acids given by Moore and Stein (1948, 1951) were checked by dividing the regression coefficient relating their concentration with optical density at 570 mμ, by the corresponding coefficient for leucine. These amino acids were purified to give correct analytical figures for carbon, hydrogen, and nitrogen, and they yielded single spots when examined by paper chromatography. Standard solutions in pH 5.0 citrate buffer were prepared containing 14 dilutions of each amino acid between 0.0005 and 0.06 mg of amino nitrogen per ml, and the colour was developed in 1-ml aliquots of these solutions as described by Moore and Stein (1948). The colour density was read in a Uvispek spectrophotometer against a blank solution containing buffer, reagent, and diluent. Where the optical density for a 1-cm light path was greater than 1.000, 1 ml of the solution was mixed with 5 ml of diluent

* 1. Abderhalden and Voitinovici (1907). 2. Marston (1928). 3. Marston (1932). 4. Vickery and Block (1930). 5. Asquith and Speakman (1952). 6. Speakman and Townend (1937). 7. Block (1939). 8. Martin and Synge (1941*a*). 9. Martin and Synge (1941*b*). 10. Martin and Synge (1941*c*). 11. Gordon, Martin, and Synge (1943). 12. Geiger (1944). 13. Lindley and Phillips (1945). 14. Lindley (1947). 15. Lindley and Drucker (personal communication). 16. Graham, Waitkoff, and Hier (1949). 17. Stakheyewa-Kaverznewa and Gavrilow (1937). 18. Vickery (1940). 19. Stewart and Rimington (1931). 20. Graff, Macula, and Graff (1937). 21. Cuthbertson and Phillips (1945). 22. Bailey (1942). 23. Rees (1946). 24. Chibnall (1942). 25. Steinhardt (1942). 26. Mizell and Harris (1943). 27. Vickery and White (1933). 28. Geiger and Harris (1942). 29. McCallum (1942). 30. Olcott (1944). 31. Astbury (1942). 32. Goddard and Michaelis (1935). 33. Goddard and Michaelis (1934). 34. Rimington (1929*a*). 35. van Slyke, Hiller, and MacFadyen (1941). 36. Rutherford, Harris, and Smith (1937). 37. Barritt (1934*a*). 38. Blackburn, Carter, and Phillips (1941). 39. Mueller (1923). 40. Elliott and Speakman (1943). 41. Harris, Mizell, and Fourt (1942). 42. Steinhardt and Harris (1940). 43. Barritt (1928). 44. Barritt (1934*b*). 45. Barritt (1927). 46. Rimington (1929*b*). 47. Tristram (1949). 48. Bailey (1937).

† Histidine figure not included.

and read against the blank similarly diluted. Table 2 shows the regression coefficients thus obtained and the ratio of these to that of leucine. The ratios agree closely with those of Moore and Stein (1948, 1951) except for glutamic acid and glycine. In subsequent work a duplicate series of standard leucine solutions was included with each analytical run. The regression coefficient calculated from these data was used to convert all the optical density readings into mg of amino nitrogen.

Table 2

COLOUR YIELDS OF AMINO ACIDS

Amino Acid	Concn. Range (mg N/ml) $(\times 10^4)$	Regression Coefficient and Standard Error	Colour Yield (Leucine = 1·00)
Alanine	7·9—79	193·9±1·9	1·00
Aspartic acid	5·3—84	185·6±0·68	0·95
	63—530	29·33±0·35	0·93
Cystine	6·0—119	105·4±0·38	0·54
Glutamic acid	4·7—75	189·4±2·3	0·97
	57—570	31·16±0·16	0·99
Glycine	9·4—94	188·8±3·8	0·97
	56—560	30·71±0·20	0·98
Leucine	5·3—63	194·5±1·4	1·00
	63—630	31·51±0·15	1·00
Valine	5·9—59	191·4±2·8	0·98
	71—589	31·57±0·13	1·00

(*e*) *Apparatus and Analytical Procedure*

The Dowex 50 columns were set up over a solenoid-operated fraction collector so that the effluent passed through a platinum wire grid before falling into a test-tube (15.4 × 1.8 cm). The momentary passage of an electric current across the grid operated an electronic counter. After the delivery of a pre-set number of drops, the solenoid moved the collector turntable forward one position.

Difficulty was experienced in maintaining the fraction size constant as the temperature of the columns was varied between 37.5 ± 0.5°C and 75 ± 1°C. Compensation could usually be made by increasing the number of drops per fraction each time the temperature was raised, so that the weight of liquid obtained, after adjustment of the pH to 5.0 for the development of colour with ninhydrin, was 1.00 ± 0.05 g. Where this could not be done, and the fractions were smaller than 0.95 g, the necessary amount of buffer was added to bring each fraction up to 1.00 g. In later experiments a cooling tube was introduced between the column tip and the conductivity grid, but this only partly resolved the difficulty. Further work has improved the accuracy of fraction measurement, and the apparatus used will be described in a forthcoming publication.

About 1 hr before use water at 37.5 ± 0.5°C (25°C for the 15-cm columns) was circulated through the column jackets and the level of the buffer above the resin was allowed to fall to within 5 mm of the resin surface. The residue obtained by evaporating 0.5 ml of the wool hydrolysate, was dissolved in 0.5 ml of pH 3.29 buffer and transferred to the top of the column. It was allowed to sink in under atmospheric pressure and three or four washings, each of 0.3 ml buffer, were then used to rinse the polythene cup and sides of the column. Finally, the column was filled to the top with buffer and connected by means of polyvinyl chloride tubing with the buffer reservoir which could be raised or lowered to control the rate of flow of the column.

The procedure followed during an analytical run was similar to that described by Moore and Stein (1951) and is summarized, together with the results of a typical analysis, in Figures 1 and 2, where the colour density at 570 mμ of each tube corrected for colour yield, is plotted against fraction number.

The identity of each amino acid peak in the figures (cf. Moore and Stein 1951) has been confirmed by paper chromatography of appropriate fractions taken from the effluent of the Dowex 50 columns. After desalting (Simmonds 1953*b*), each fraction was chromatographed with and without the appropriate amino acid as a marker. In each case only one spot was observed on the paper chromatograms after spraying with ninhydrin. The only peaks of doubtful identity are those labelled unknown (1) and unknown (2) in Figure 1.

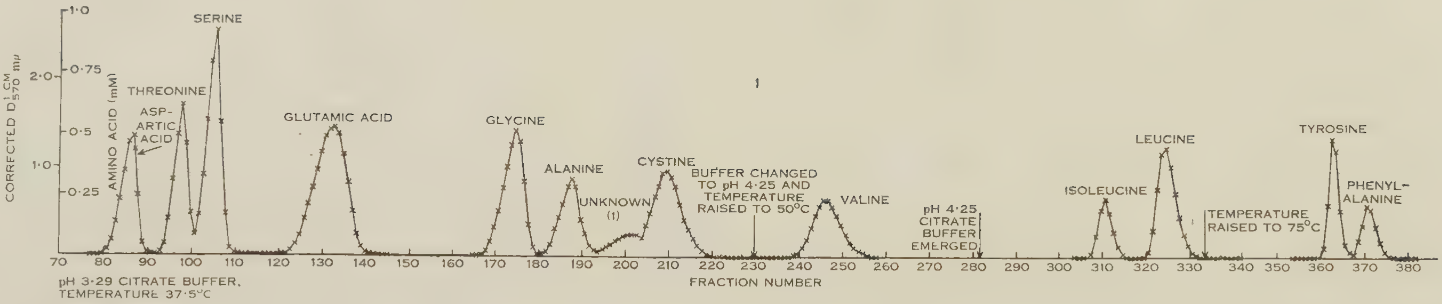
(*f*) *Estimation of Other Amino Acids*

(i) *Cystine*.—Since the colour yield of cystine is low (Moore and Stein 1948) and it tends to emerge from the column as a broad plateau it was estimated in a separate sample of the same wool by the method of Shinohara (1935, 1937). The results of these analyses are included in Table 3.

(ii) *Methionine*.—As thiodiglycol was not available as an anti-oxidant, methionine was determined by the volatile iodide method of Baernstein (1932, 1936).

(iii) *Tryptophan*.—This amino acid was estimated spectrophotometrically by the method of Goodwin and Morton (1946). A sample of wool (30 mg) was dissolved by warming for 10 min in 10 ml 1N NaOH. The clear solution was cooled and diluted to 100 ml with water. Solutions containing 10 ml 1N NaOH alone and together with 10 mg tryptophan were treated similarly and no loss of tryptophan was observed in the latter. The samples were read in a Beckmann spectrophotometer at 280, 294.4, 340, and 370 mμ, using 0.1N NaOH as a blank.

(iv) *Proline*.—Contrary to the experience of Moore and Stein (1951) glutamic acid and proline were not resolved sufficiently in the Dowex 50 column effluents to permit accurate estimation of the latter with ninhydrin at pH 5.0. Proline was therefore estimated by conducting the ninhydrin reaction at pH 1 in the presence of phosphoric and acetic acids (Chinard 1952). The reagent was freshly prepared as required, by dissolving 2.5 g of ninhydrin in a mixture of 40 ml 6M H_3PO_4 and 160 ml glacial acetic acid. Aliquots (2 ml) of the



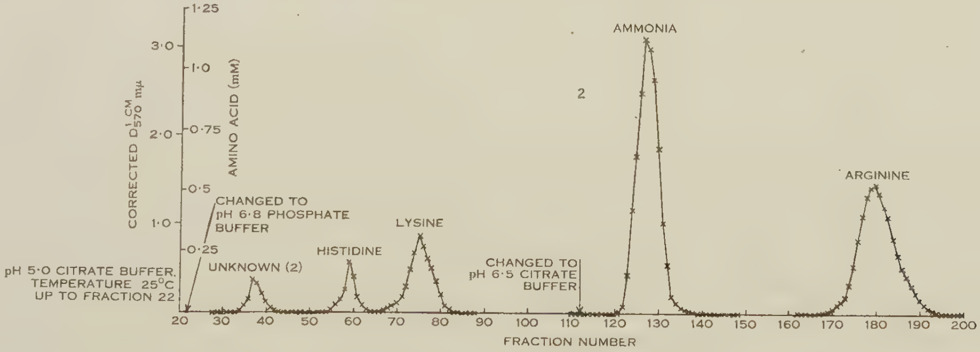


Fig. 1.—Separation on a 100-cm Dowex 50 column of acidic and neutral amino acids in hydrolysate of 64's quality merino wool. Column loading 0.750 mg N.

Fig. 2.—Separation on a 15-cm Dowex 50 column of basic amino acids in hydrolysate of 64's quality merino wool. Column loading 1.658 mg N.

proline-containing solutions were adjusted with 2N NaOH to the first pink colour of phenolphthalein, and then with 0.1N HCl until the indicator was just colourless. Sodium nitrite (6M, 2 ml) and glacial acetic acid (1 ml) were added and the deamination was allowed to proceed according to the method of Troll (1953). After evaporating to a small volume three times with 5 ml conc. HCl, the residue was neutralized as before with 2N NaOH and 0.1N HCl, 5 ml 6M H_3PO_4 and 5 ml glacial acetic acid were added, and the solution was diluted to 50 ml with distilled water. Aliquots of this solution (0.5, 1.0, 1.5, and 2.0 ml) were withdrawn for analysis and made up with a deaminated mixture of 14 amino acids (excluding proline) to 2.0 ml total volume. To each aliquot, reagent (4 ml), was added and the solutions were heated in a boiling water-bath for 30 min—the optimum time for colour development. The tubes were diluted with glacial acetic acid (4 ml) and were read in a Uvispek spectrophotometer at 515 mμ.

Samples of the original wool hydrolysate, a standard solution containing 0.70 mg of proline per ml, and a reagent blank consisting of a mixture of 14 amino acids (excluding proline) in the proportions in which they occur in wool, were taken through the procedure outlined above.

(*g*) *Calculation of Results*

(i) *Amino Acids in the Effluent of the Dowex* 50 *Columns.*—The optical density readings obtained for each amino acid were summed, converted into leucine equivalents by multiplying by the reciprocal of the colour yield, and divided by the regression coefficient obtained from the standard leucine control series. The results were expressed as a percentage of the nitrogen determined on an aliquot of the hydrolysate by the micro-Kjeldahl method.

(ii) *Tyrosine and Tryptophan.*—The total quantity of tyrosine and tryptophan present was calculated from the optical density reading at 294.4 mμ, the point of intersection of the absorption curves of these two amino acids. From the reading at 280 mμ the amount of each amino acid was obtained using the equation given by Goodwin and Morton (1946).

(iii) *Proline.*—Calculation of the percentage of proline nitrogen present was carried out by the slope ratio assay method (Clarke 1952).

(*h*) *Results*

Table 3 summarizes the results obtained in the present investigation, together with the standard error associated with the mean in each case.

III. DISCUSSION

The wool fibre consists of at least three distinct morphological components (Lindberg *et al.* 1949) and is therefore almost certainly not a homogeneous protein. Chemical (Goddard and Michaelis 1935; Alexander 1951; Horio and Kondo 1953; Mercer 1953; Fraser and Rogers 1953), electrophoretic (Gillespie

Table 3

AMINO ACID COMPOSITION OF MERINO 64'S QUALITY WOOL

Carbon (dry weight) 50·23%; hydrogen 8·13%; nitrogen 16·62%; sulphur (dry weight) 3·68%; ash 0·01%; moisture 11·55%

Amino Acid	No. of Analyses	Mean of Total Nitrogen and Standard Error (%)	Weight from 100 g Dry Wool	Weight Residues in 100 g Dry Wool	G-equiv./ 10^4 g Dry Wool
Alanine	7	3·51±0·08	3·71	2·96	4·17
Arginine	4	20·32±0·04	10·49	9·40	6·02
Aspartic acid	8	4·24±0·18	6·69	5·78	5·03
Amide N	4	7·46±0·57**	1·42	1·42	8·86
Cystine	11 (5)	7·93*(4·95)±0·05 (±0·19)	11·30	10·45	4·71
Glutamic acid	7	8·58±0·13	14·98	13·15	10·18
Glycine	7	5·80±0·09	5·16	3·92	6·88
Histidine	5	1·46±0·10	0·90	0·80	0·57
Isoleucine	8	1·97±0·05	3·07	2·65	2·34
Leucine	8	4·90±0·12	7·63	6·58	5·82
Lysine	5	3·25±0·15	2·82	2·47	1·93
Methionine	2	0·39‡±0·02	0·69	0·61	0·46
Phenylalanine	6	1·75±0·09	3·43	3·06	2·08
Proline	2	5·33§±0·05	7·28	6·14	6·33
Serine	8	7·25±0·19	9·04	7·50	8·61
Threonine	8	4·61±0·13	6·55	5·56	5·47
Tryptophan	4	1·73‖±0·12	2·10	1·91	1·03
Tyrosine	5	2·97 (3·08‖)±0·08	6·38	5·75	3·53
Unknown (1)	5	1·18 (2·22¶)±0·10	—	—	—
Unknown (2)	4	0·71†±0·07	0·68	0·60	0·42
Valine	7	3·57±0·10	4·96	4·20	4·24

* Estimated by method of Shinohara (1935, 1937).

† Assumed to be hydroxylysine with a colour yield the same as lysine.

‡ Estimated by method of Baernstein (1932, 1936).

§ Estimated by method of Chinard (1952).

‖ Estimated by method of Goodwin and Morton (1946).

¶ Corrected for colour yield of cystine (see discussion).

** Uncorrected for decomposition of serine and threonine during hydrolysis.

and Lennox 1953), molecular weight (Ward 1952; Friend and O'Donnell 1953), and end-group studies (Middlebrook 1951) further emphasize its heterogeneity. Amino acid analyses such as those presented above are mainly of value therefore in permitting a comparison between the overall composition of different types of wool. However, it may be pertinent to discuss the similarities and differences observed between the results obtained in this and previous investigations.

In general the results confirm those regarded as reliable in the literature (references 5-15, Table 1) but they are somewhat lower than those reported by Graham, Waitkoff, and Hier (1949) (reference 16, Table 1). The major differences lie in the values reported for glycine, and the ratio of leucine to isoleucine (Simmonds 1953*a*). The only analysis for glycine previously reported (7.9 per cent. nitrogen (Block 1939)) is substantially higher than the figure of 5.8 per cent. recorded in the present investigation.

Speakman and co-workers (Speakman and Townend 1937; Asquith and Speakman 1952) have carried out analyses for the acidic and basic amino acids of wool to determine whether the sum of the free carboxyl groups of glutamic and aspartic acids is equivalent to that of the free basic groups of arginine, histidine, and lysine. The data in Table 3 support the conclusions of Speakman and co-workers in indicating that this is approximately so, provided that tyrosine is included with the acidic amino acids (cf. Steinhardt and Harris 1940). The figures below are expressed as gram-equivalents of free acidic and basic groups in 10^4 g of wool.

Free Carboxyl Groups		Free Basic Groups	
Glutamic acid	10.18	Unknown (2) (see footnote to Table 3)	0.71
Aspartic acid	5.03	Lysine	1.93
Tyrosine — OH	3.53	Arginine	6.02
		Histidine	0.57
		Amide	8.86
	18.74		18.09

The results shown in Table 3 indicate that the amount of nitrogen unaccounted for in the present investigation is small. The only two peaks of doubtful identity, labelled unknown (1) and unknown (2) in Figure 1, are being currently investigated.

The only element which has not been completely accounted for is sulphur. The figures for cystine sulphur (3.02 per cent. dry weight) and methionine sulphur (0.15 per cent. dry weight) fall short of the total sulphur estimated (3.68 per cent.) by 0.51 per cent. A similar discrepancy in the sulphur analyses has been reported by Barritt (1934*a*), Bailey (1937), Schöberl and Rambacher (1940), Schöberl (1952), Cuthbertson and Phillips (1945), and Marston (1946). In the present work, part of the missing sulphur was present as cysteic acid and the peak corresponding to this constituent was observed in the effluent of the Dowex 50 columns preceding the emergence of aspartic acid. However, there was insufficient cysteic acid or any other unidentified component to account for the remaining sulphur as a sulphur-containing amino acid. The unidentified sulphur is also unlikely to be present as sulphate since the ash content of the wool is low and the method used for the determination of total S (Grote and Krekeler 1933) does not estimate sulphur in the form of sulphate.

A further discrepancy arises when the percentage of cystine calculated by the Shinohara (1935, 1937) and the Moore and Stein (1951) procedures are compared (see Table 3). Because of the small peak of unknown (1) preceding it, and the sometimes irregular nature of the cystine peak, it was difficult to estimate the latter as accurately as the other amino acids. A low recovery of cystine has similarly been observed by Stein (1953). The Shinohara technique may therefore estimate reducing material other than cysteine, or the cystine may be racemized or destroyed during the 16 hr hydrolysis to which the wool sample is subjected before Moore and Stein analysis. Toennies and Bennett (1935) have discussed the extent of racemization of cystine occurring during acid hydrolysis, and since the DL- and L- forms differ considerably in solubility it is possible that unknown (1) is one stereoisomeric form of cystine partially separated from the other. If this were so, the total cystine content would be 7.17 per cent., which is in closer agreement with the figure obtained by the Shinohara method. When a synthetic mixture of amino acids, simulating the composition of wool but not subjected to acid hydrolysis, was examined by the Moore and Stein procedure, the cystine peak was observed to be perfectly regular and it accounted quantitatively for the cystine known to be present.

IV. ACKNOWLEDGMENTS

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THE INHERITANCE OF ANTIBODY RESPONSE TO TOBACCO MOSAIC VIRUS IN RABBITS

By W. R. Sobey*

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Summary

In a crossbred population of rabbits it was found that the level of secondary antibody response to a routine injection of T.M.V. was highly heritable ($h^2 = 0.876$, S.E. 0.09).

I. Introduction

It is common experience among immunologists that animals of the same species vary widely in their ability to produce antibodies. Comparisons between the responses of different inbred lines indicate that at least some of the variability is genetic, as shown in guinea pigs by Lewis and Loomis (1928), in rabbits by Lurie (1941), and in mice by Gorer and Schutze (1938), Davidson and Stern (1949), and Fink and Quinn (1953). Little work has been done to study the inheritance of response. Kleczkowski (1939) presented suggestive evidence for a single gene control over the level of response to small injections of human serum into rabbits, but the data are limited. Scheibel (1943) when selecting for and against the ability to produce antitoxin to a standard dose of diphtheria toxin in guinea pigs, obtained good evidence of a single gene control over the ability to 'produce' or 'not produce' antitoxin. There was little or no evidence, however, of a genetic control of the level of response of the 'producers.' Carlinfanti (1948) measured the levels of human isoantibodies anti-A_1 and anti-B quantitatively in 51 families with 159 children, and found them to be almost entirely genetically determined. This interesting study does not, however, contribute information to the inheritance of response to introduced antigens; the presence of isoantibodies not having been satisfactorily explained as yet.

The results described in this paper are an approach to the quantitative study of the inheritance of antibody response.

II. Experimental

(*a*) *Antigen*

Tobacco mosaic virus (T.M.V.) was selected as a suitable antigen. This antigen has an extremely high molecular weight: 33,000,000 (Schramm and Friedrich-Freska 1941), 50,000,000 (Kausche and Ruska 1939); and is strongly

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antigenic. The T.M.V. used in the present work was kindly supplied by Dr. A. Kleczkowski of Rothamsted Experimental Station. Injections were given by the intravenous route to avoid the possibility of introducing variables independent of antibody response, e.g. skin thickness or local blood supply. Each rabbit received a total dose of 0.5 mg T.M.V. given in two injections, each consisting of 2 ml 0.0125 per cent. T.M.V., with an interval of a week between injections. Rabbits were injected in the left ear and bled from the right ear, the marginal ear vein being used in both operations. Bleedings were made on the tenth and thirteenth days after the last injection.

(*b*) *Antibody Response*

The equivalence zone method was used throughout. Some preliminary tests were made to find the range of response at the injection dose selected. The method, as it was finally adopted and used throughout the experiment, is given below. Eight 5-ml tubes were set up for each serum to be tested and 2.5 ml of the appropriate antigen dilution was added to each (Table 1).

TABLE 1

EXPERIMENTAL SCHEME ADOPTED

Tube No.	2·5 ml T.M.V. (% dilution)
1	0·04
2	0·02
3	0·01
4	0·005
5	0·0025
6	0·00125
7	0·000625
8	0·0003125

To each tube was then added ½ ml of test serum diluted by half. The tubes were incubated in a 40°C water-bath for half an hour and left overnight in the refrigerator. They were then centrifuged and the supernates removed and divided in two, half into each of two tubes and the tube number retained for each pair of tubes. To one half was added 0.5 ml of strong anti-serum diluted by half, and to the other 0.5 ml 0.01 per cent. T.M.V. These were then placed in the water-bath and the equivalence zone estimation read when precipitation was complete. The zone is indicated by a pair of tubes in which no precipitation occurs, i.e. there is neither excess antigen nor antibody. Where the zone was wide, or overlapping, the estimate of response was obtained by interpolation. The scale of values measuring antibody response is given in Table 2.

The antigen dilutions are in a geometric progression and the tube number for any equivalence estimation represents the inverse log of the antibody response. The extremes, 10 and 1, are not strictly to scale, but they cover the present range of response adequately. This method of measuring the response is convenient and also useful in view of the evidence of Carlinfanti (1948) and Holt (1951) that the scale of antibody response is logarithmic.

TABLE 2

SCALE OF VALUES

Scale	10	9	8	7	6	5	4	3	2	1
Tube No.	>1	1	2	3	4	5	6	7	8	<8

The secondary time response curves, as illustrated in Table 3, all reach a peak by 7 days, after which they fall off very gradually.

TABLE 3

SECONDARY TIME RESPONSE CURVES

Serial No.	Response at the Following Days After Last Injection						
	2	4	7	10	13	17	20
399	5	5	5·5	5·5	5		
400	4	4·5	6	5	5		
401	3	4	4·5	4	4·5		
402	1·5	4·5	5·5	5·5	5·5		
403	1·5	5	5	5	5		
404	2	5	6	5	5		
405	1	1	1	1	1		
406	1	1	1	1	1		
407	1	1	1	1	1		
408	1	1·5	2	2	2		
409	1	1	2·5	2	2		
410	4·5	6	7	7	6	5	5
411	5	7	8	7	6	5·5	5·5
412	3·5	4·5	4	5	4·5	5	4·5

The estimated experimental error of measurement is ± 0.6 when calculated on the assumption that the variance of the difference between 10- and 13-day responses is twice the experimental error for any individual observation. Within the limits of this error the order of ranking can be regarded as being the same whether judged by response at 4, 7, 10, or 13 days. The variation is greatest at 7 days but only slightly reduced at 10 and 13 days, and these were selected for the present investigation. By taking two measurements it was possible to estimate the experimental error of measurement and to limit the probability of missing any unexpected variability of time response. All calculations relating to heritability are based on the response at 10 days.

(*c*) *Rabbits*

The parent-offspring comparisons include only measurements made on the F_1 and F_2 of a Flemish Giant (F.G.), Ermine Rex (E.R.) cross. Some measurements were made on E.R. rabbits and these are included for purposes of comparison and frequency distribution only.

The animals were housed in ventilated, unheated buildings throughout the year. Up to 9 months of age animals were fed pellets and thereafter a mixture of oats and pellets. All animals were injected at between 5 and 6 months of age, at which age they reach sexual maturity, and antibodies passively acquired from the mother will have disappeared. The pre-injection sera of 25 rabbits, whose dams had received the routine injection of antigen before being mated, showed no demonstrable passive antibodies to the antigen.

III. RESULTS

Measurements made in the parent generation included an unselected F_1 population of F.G. × E.R. cross, and an unselected population of E.R. rabbits. The frequency distributions of response of these two populations are given in Figure 1*A*,*B*.

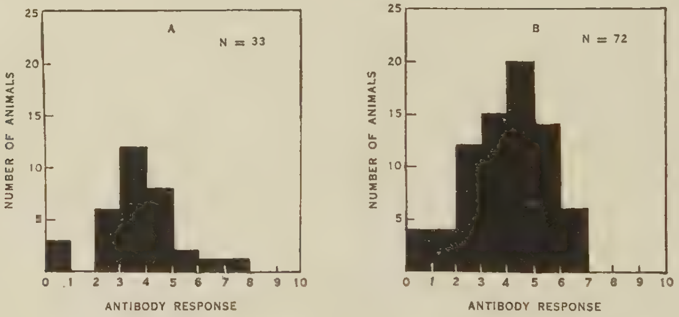

Fig. 1.—Frequency distribution of secondary response to routine injection of T.M.V. in two populations of rabbits. *A*, an E.R. population ($\bar{x} = 4.12$), and *B*, an E.R. × F.G. cross population ($\bar{x} = 4.57$). The means are not significantly different ($t_{103} = 1.49$).

In view of possible genetic differences between the populations, these distributions were compared statistically. They show a variance ratio of 1.185, which is not significantly different at the 5 per cent. level and justifies a comparison of their means by a *t* test. This gave $t_{103} = 1.49$, which is not significant at the 10 per cent. level, and the two populations can be regarded as being the same in their response to T.M.V. To increase the data on the frequency distribution of response the data from the two populations were added; this result is shown in Figure 2.

This distribution is obviously normal, even to the extent of being almost suspect, and justifies the use of a logarithmic scale in the measurement of response.

The heritability of response to T.M.V. was measured by a mid-parent offspring regression; 213 animals, comprising 32 litters, were measured. This regression, shown in Figure 3, gave a heritability $h^2 = 0.876$, S.E. 0.09.

In spite of the relatively low number of points making up the regression, these data clearly demonstrate the secondary response to T.M.V. to be highly heritable.

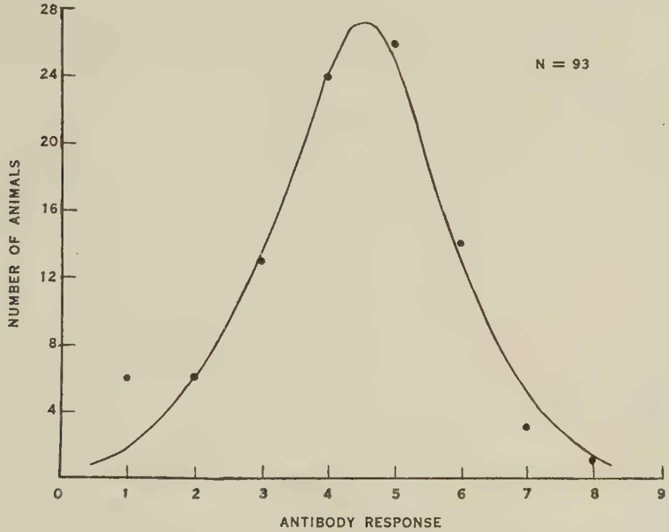

Fig. 2.—Frequency distribution from the combined data in Figure 1. $\bar{x} = 4.43$, $s\bar{x} = 0.14$, $s = 1.44$. Coefficient of variation = 32.5 per cent.

IV. DISCUSSION

These results indicate that ability to produce antibodies to T.M.V. is highly heritable and it would be a relatively simple matter to select lines of animals with a high or low secondary response to this antigen. It was shown by Sobey (1953) and Sang and Sobey (1953), using a double diffusion technique in agar, that under the conditions prevailing in the present work, T.M.V. was apparently acting as a single antigenic fraction and producing a single specific antibody response. It was further shown that with a more prolonged antigenic stimulus T.M.V. elicited more than one antibody response; these could not be individually distinguished by the routine equivalence zone method used but only by the more delicate technique of agar diffusion. Thus had a greater antigenic stimulus been employed as a routine, the method of estimating antibody response would still have given a single measurement of what was in fact more than one antibody response. There are no grounds for assuming that these responses would be correlated, and if they were not, the resulting confusion in measurement would be likely to give a low or non-significant measure of heritability. Nevertheless, the individual antibody responses could be as completely under genetical control as that found to the single antigenic T.M.V. fraction in this study.

These arguments raise fundamental issues regarding any study of the significance of antibodies in disease resistance. Pathogens generally are antigenically complex and may be expected to stimulate more than one specific antibody response. Of these antibodies it is often only one which plays a major part in immunity; in pneumococcal infection antibodies to the specific polysacchonides afford a high degree of specific immunity, whereas those to the nucleoprotein antigens have little protective value (Topley 1929; Topley and Wilson 1948; Bailley 1950). The evidence of Felix (1924) indicates that in motile bacilli the antibodies to flagella antigens play little part in immunity, whereas those to the somatic antigens are of great importance.

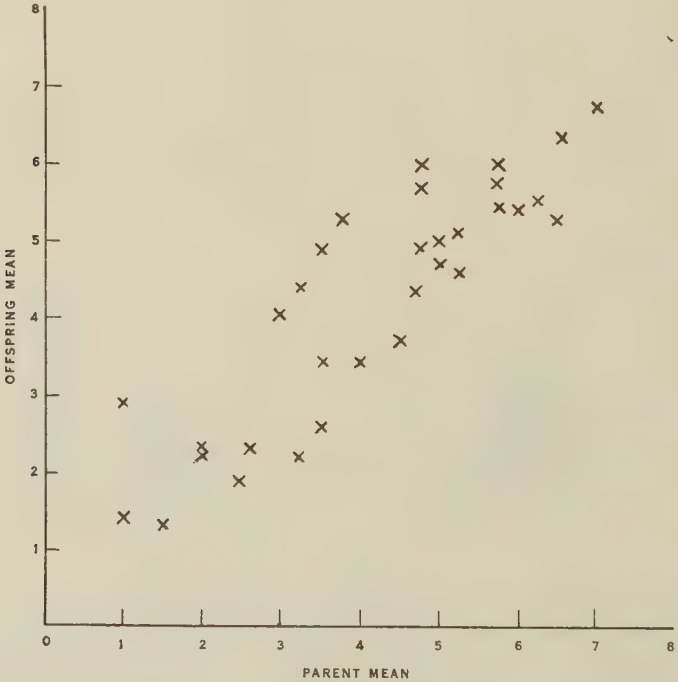

Fig. 3.—The regression of mean secondary response of offspring on mean secondary response of parents to routine injection of T.M.V. 213 Animals, comprising 32 litters, were measured. The estimated heritability was $h^2 = 0.876$, S.E. 0.09.

The relationship between the quantitative antibody response to the specific antigens concerned in resistance and the degree of resistance is not yet fully understood. There is evidence of a positive correlation (Smith 1932; Carlinfanti and Cavalli 1945; Bailley 1950), but this does not always justify the acceptance of a causal relation (Gorer and Schutze 1938; Weir, Cooper, and Clark 1953). Another factor concerning the individual antibody responses which has received little attention is the variability of their time responses, and whether or not speed of reaction is concerned in resistance.

To ensure a clear understanding of the inheritance of antibody response and of the relation of antibodies in resistance, it would appear necessary to study in detail the individual antibody responses involved. Measurements failing to differentiate the individual antibody responses are likely to be misleading in any quantitative study involving complex antigens.

If specific antibody responses are generally as highly heritable as that demonstrated with T.M.V., it might be possible to select lines of animals with a high or low antibody response to each of the individual antigenic fractions of a pathogen, provided these could be measured. Such lines of animals would afford useful material for the study of the problems raised.

V. Acknowledgments

I should like to thank Professor C. H. Waddington, Institute of Animal Genetics, Edinburgh, for the facilities, and the C.S.I.R.O. for the finance, to undertake this study. I am indebted to Dr. J. M. Rendel, Dr. A. Robertson, Dr. A. Kleczkowski, and Dr. J. Sang for their advice and interest.

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OPTIMUM CONDITIONS FOR THE LOCAL (INTRAVAGINAL) ACTION OF OESTROGENS

By J. D. Biggers* and P. J. Claringbold*

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Summary

The optimum conditions for the intravaginal administration of oestrogens are obtained by the use of 1 per cent. aqueous egg albumin as solvent, and two or four injections spread over 24-36 hr. The administration of oestrone, oestradiol-3,17β, and diethylstilboestrol in distilled water by four injections in 36 hr gives a close approximation to the optimum. Oestriol, however, has considerably reduced activity when administered under these conditions.

Relative activities of oestrone, oestradiol-3,17β, oestriol, equilin, equilenin, and diethylstilboestrol have been determined, using four injections in 36 hr, and 1 per cent. aqueous egg albumin as solvent. Under these conditions all have the same activity as oestrone, except oestradiol-3,17β, which is approximately 1.5 times as active.

The optimum conditions for intravaginal administration are similar to those for subcutaneous administration with respect to the period over which injections are spread. The two techniques, however, differ with respect to the frequency of injection; while repeated subdivision of dose in the subcutaneous method increases response there is a maximum response reached by four injections with the intravaginal method.

The relation of the above results to the quantitative study of oestrogen activity and the local action of oestrogens on the vaginal epithelium are discussed.

I. Introduction

Marrian and Parkes (1929) studied several variables in the subcutaneous Allen-Doisy technique for oestrogen assay. Using four injections they found that maximum sensitivity was elicited by spacing the injections over 24-36 hr, and that even greater sensitivity was obtained by administering the total dose in many injections (up to 20). Recently attention has been given to the technique of intravaginal assay. Mühlbock (1940) found, using oestrone, that three injections resulted in greater sensitivity than either one or two injections, while Emmens (1941), using oestrone, oestradiol-3,17β, oestriol, and diethylstilboestrol, showed that two injections in 24 hr resulted in greater sensitivity than a single injection. Since then, two injections in 24 hr have been accepted as standard (Emmens 1950*a*, 1950*b*; Biggers 1953*a*). Recently, however, Sulman (1952) has claimed that greatly increased activity is obtained if five injections are spread over 36 hr. In view of the incompleteness of the previous work on the intravaginal technique, systematic investigations of variations of technique were

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undertaken, with the object of determining optimum conditions of administration. Experiments of factorial design which permit the study of factor interactions, and which also afford economy in animals and time, were employed where possible.

II. Materials and Methods

Four colonies, of 260, 360, 380, and 440 albino mice bred in this department, were ovariectomized. Their management was identical with that described by Biggers (1951), tests being carried out at 14-day intervals.

The oestrogens were administered either in distilled water or in 1 per cent. aqueous egg albumin solution. The solutions, with the exception of those used in experiments on the effect of volume, were prepared as described in earlier papers (Biggers 1951, 1953*b*) so that the volume of each injection was 0.01 ml. The oestrone, oestradiol-3,17β, and diethylstilboestrol were obtained from Organon Laboratories, the oestriol from Parke Davis & Co., and the equilin and equilenin from Ayerst, McKenna and Harrison Co.

III. Design and Analysis of Experiments

Past experience had indicated that under the various treatment combinations to be employed wide variations in the level of response would occur. Where necessary pilot experiments were used to locate the dose response lines, and on the basis of these results factorial experiments were designed (see Fisher 1949). In certain instances parallelogram designs have been adopted in order to avoid large regions of 0 and 100 per cent. response. All experiments have been analysed using the angular transformation and analysis of variance, according to the methods described by Claringbold, Biggers, and Emmens (1953).

Since several variables have been investigated simultaneously in each experiment, discussion has been facilitated by placing the design, results, and analyses of each experiment in an appendix.

In the text all expressions of the form "regression of response on . . ." should be taken to mean "regression of the angle of response on the logarithm of . . ." To facilitate computation the various experiments are calculated in the logarithmic base determined by the treatment levels, and in order to compare the various experiments the parameters concerned have been transformed into logarithms to base 10. Fiducial limits ($P = 0.05$), where required, have been placed between braces, after the estimates of a parameter; standard errors are indicated by placing them after the ± sign.

IV. Results

(*a*) *Total Volume of Injection* (*Table* 4)

Oestrone, administered in distilled water, has been used in this investigation. The full analysis of variance shows significant linear ($P < 0.001$) and quadratic ($0.05 > P > 0.01$) components of the regression of response on volume. A separate analysis of the first three points, however, shows no differences between them, and thus the regression is due to small responses obtained with the largest volumes. The effect of volume of injection is shown schematically in Figure 1, where it is seen that, provided the total volume of the two injections does not exceed 0.02 ml, the response is maximal. Volumes greater that this are highly inefficient, owing to leakage of the solution from the vagina. In all subsequent work a volume of 0.01 ml per injection has been employed. Both Mühlbock (1941) and Emmens (1950*b*) used this volume, while Sulman (1952) used a variable volume and found that in selected animals volumes of 0.02 ml may be used.

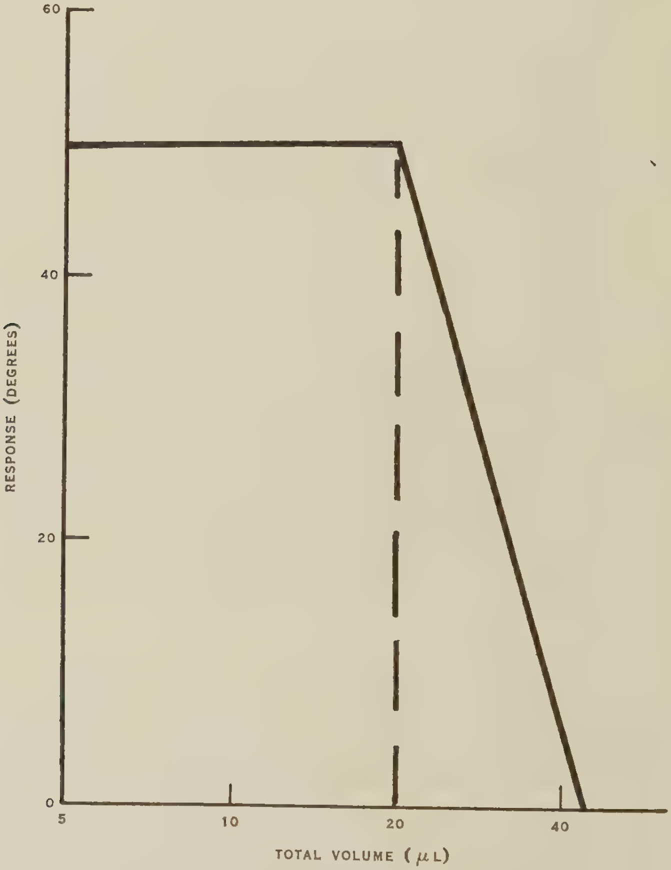

Fig. 1.—Effect of volume of injection on the response of mice to a dose of oestrone.

(*b*) *Frequency of Injection* (*Tables* 5-9)

The effect of frequency of injection (2, 4, and 8 injections) during various time intervals has been studied using oestrone, oestradiol-3,17β, and diethylstilboestrol, administered in distilled water. In the analysis of variance for all experiments no significant interactions were observed between frequency of injection and other variables, and hence the effect of frequency can be discussed independently.

Oestrone and oestradiol-3,17β.—For both the analysis of variance shows the linear components of the regressions of response on frequency as non-significant while the quadratic components are highly significant. This implies that there are no significant differences in response to two injections and eight injections, whereas four injections are more effective. The effect was consistent over all time intervals and doses studied.

Diethylstilboestrol.—Here the linear and quadratic components of the regression of response on frequency are both significant. Eight injections are much less effective than two injections, both being less effective than four injections. This relationship is found over all time intervals and doses studied.

Oestriol.—This oestrogen was found to behave in an analogous manner to oestrone and oestradiol-3,17β, in the restricted time interval (24 hr) studied. In terms of oestrone as standard, the relative activity of oestriol, when administered in distilled water by four injections in 24 hr (Table 2), shows that oestriol is considerably less active under these conditions than the three oestrogens mentioned above. Therefore no further investigations on oestriol in distilled water were undertaken.

(*c*) *Time Interval* (*Tables* 6-8, 10-12)

In all the following experiments distilled water was again the vehicle of administration.

Oestrone.—In an initial experiment with oestrone a two-injection technique was employed over time intervals ranging from 0.9 to 24 hr (Table 10), and a highly significant linear regression of response on time interval ($b_1{}^*$) was observed. As the interval between the two injections was increased the response became greater. A factorial experiment was designed (Table 6) investigating time intervals of 16, 24, and 36 hr in conjunction with other variables, and the linear regression observed above was confirmed. In yet another experiment (Table 11) this effect has been confirmed over the time intervals of 13.5, 18, and 24 hr.

The various regressions, and their standard errors, have been converted to common logarithms and a homogeneity χ^2 test applied. The weighted mean regression was 42.7 ± 6.1 ($\chi^2_{[2]} = 3.20$, $0.3 > P > 0.2$).

Further investigation with four injections over the time intervals 24, 36, and 48 hr (Table 12) showed that 36 hr gave maximum activity. The collective information is shown schematically in Figure 2.

Oestradiol-3,17β.—A factorial experiment was designed on the basis of the results obtained with oestrone (Table 7). However, in contrast to these findings, no evidence of a regression of response on time interval ($b_1{}^*$) was found with oestradiol-3,17β. In a further experiment (Table 11), using four injections, the responses over time intervals 13.5, 18, and 24 hr were studied, and no evidence of a linear regression was found in this range. The quadratic effect (Table 11) is difficult to explain and would require further work for its confirmation. The effect has not been examined further as it is of no particular interest in the present work. The experiments reported, however, fully demonstrate a difference in the action of oestrone and oestradiol-3,17β. The data are illustrated in Figure 2.

Diethylstilboestrol.—Similar experiments to the above were designed for diethylstilboestrol with appropriate modifications in dosage (Tables 8 and 11). No significant differences in response were observed over the time intervals 16-36 hr, while in the 13.5-24 hr interval a steep linear regression (b_1^*) was obtained. The linear regression coefficient, in common logarithms, is 190 ± 41. This is significantly greater than the corresponding regression coefficient for oestrone ($t[\infty] = 3.61$, $P < 0.001$). The data are illustrated in Figure 2.

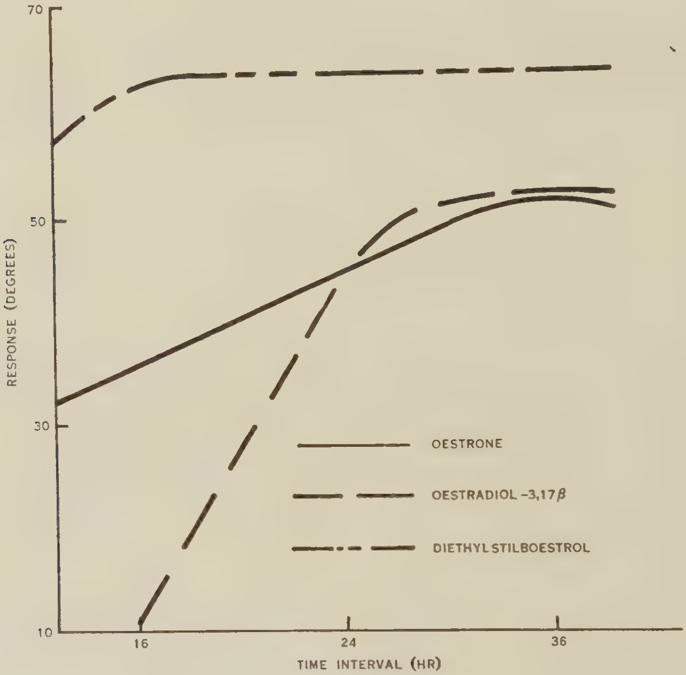

Fig. 2.—Effect of time interval over which the injections are spread on the response of mice to oestrone, oestradiol-3,17β, and diethylstilboestrol.

(*d*) *Unequally Divided Doses* (*Table* 13)

In the following experiment the effect of different dosage of oestrone *per injection* by a four-injection technique over 36 hr was examined. Four dose levels were investigated and at each dose level five different treatments were used. The design is illustrated in Figure 3. The treatments have been coded (– 2, – 1, 0, 1, 2) in equal steps for the purposes of analysis, this being possible since the distribution of total dose fractions has been arranged in a linear order.

Treatment (0) thus represents the normal four-injection technique, i.e. four equal injections.

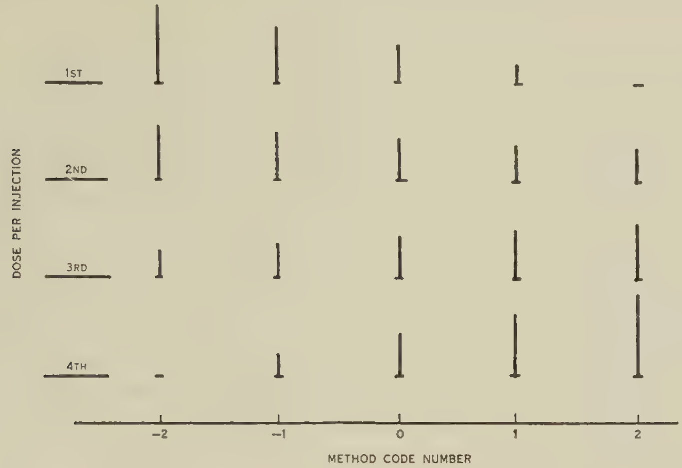

Fig. 3.—Scheme of doses used in the experiment designed to investigate the effect of unequal division of total dosage.

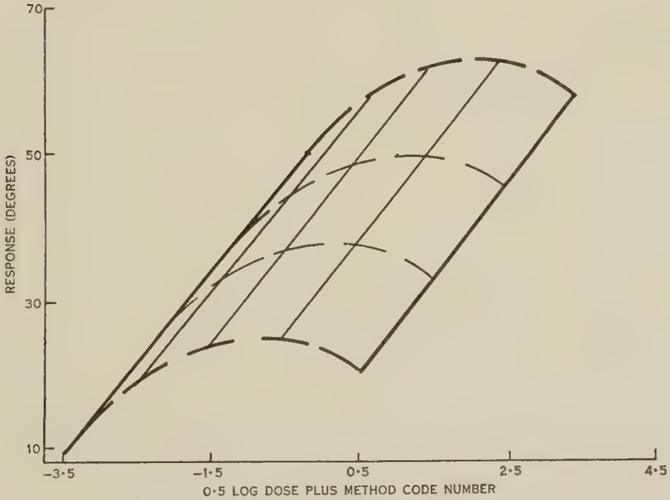

Fig. 4.—Effect of unequal doses *per injection* on the response of mice to oestrone. (See Finney (1952) for explanation of this method of plotting results.)

The results of the experiments are shown in Table 13. The analysis of variance shows that both linear and quadratic $(0.01 > P > 0.001)$ components of regression of response on treatments are significant. Figure 4 has been plotted from the calculated regression coefficients. Inspection of the figure shows that treatment (1) was slightly more effective than any other, indicating that a moderately increasing series of doses is more effective than equal doses, and considerably more effective than any other treatment.

(*e*) *Studies with Egg Albumin*

Previous studies have shown that with a two-injection technique the potency of oestrogens is enhanced when administered in 1 per cent. aqueous egg albumin solution (Biggers 1951, 1953*b*). It was suggested that the protein bound the oestrogen, and this prevented its rapid removal from the vagina by absorption into the blood stream. The results presented above show that four injections in 36 hr is the technique by which oestrogens should be administered for optimum activity. This fact may be dependent upon the normal rate of utilization of oestrogen, and under these conditions losses may be expected to be minimal. If these hypotheses are true we may predict that egg albumin as a solvent will have little effect in enhancing the activity of oestrogens administered over 36 hr. The experiment shown in Table 12 was designed to test this prediction. Oestrone and a four-injection technique were used over the time intervals 26, 36, and 49 hr.

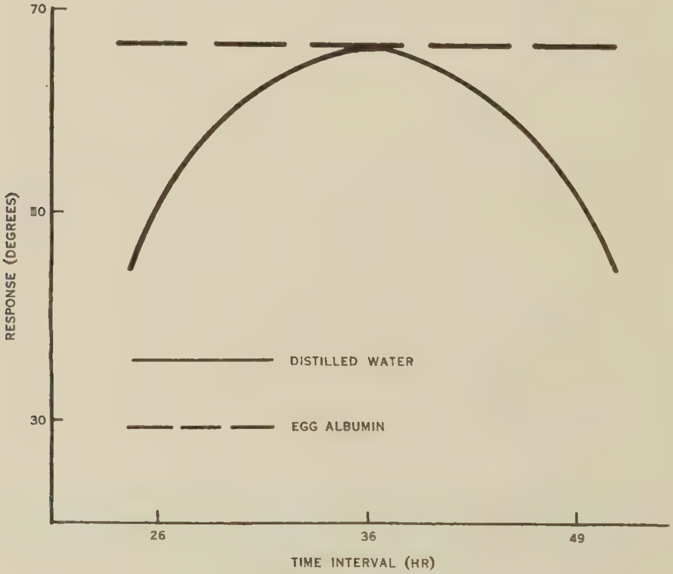

Fig. 5.—Effect of distilled water and 1 per cent. aqueous egg albumin as solvents, at various time intervals over which the injections are spread, on the response of mice to oestrone.

The analysis of variance shows a significant *vehicle* × *time interval* interaction. On partitioning of the sums of squares the quadratic component only is significant ($0.05 > P > 0.01$). Further analysis shows no linear or quadratic regression of response on time interval in the egg albumin data. Both the above quadratic effects are therefore due to the small responses at 26 and 49 hr, with a clear optimum at 36 hr. A comparison of the pooled median effective doses (M.E.D.) of the three egg albumin dose response lines and the M.E.D. of the dose response line obtained with distilled water at 36 hr shows no difference between them ($t_{[1]} = 0.02$, $P > 0.05$). The results are illustrated in Figure 5.

Relative activity of oestrone in distilled water and in 1 *per cent. aqueous egg albumin by a one-injection technique.*—Table 1 shows the M.E.D. ratios obtained with oestrone administered by one injection in distilled water and in 1 per cent. aqueous egg albumin. The M.E.D. of each line shows how profoundly the albumin enhances the activity of the oestrogen. These two lines were determined on different occasions, but the effect has not been examined more critically by simultaneous determinations since the difference is far greater than the secular shifts in sensitivity normally observed in the colonies (Biggers 1953*a*). The ineffectiveness of oestrone by a one-injection technique in distilled water is in agreement with the insensitivity observed earlier by Emmens (1941), using 50 per cent. aqueous glycerol as solvent.

TABLE 1

MEDIAN EFFECTIVE DOSES ($10^{-4} \mu g$) AND THEIR RATIOS FOR TWO TECHNIQUES OF ADMINISTRATION

Technique	Test	Solvent		Ratio and its Fiducial Limits ($P = 0 \cdot 05$)
		Distilled Water	1% Aqueous Egg Albumin	
One injection	1	178	25·8	6·90 {3·91-12·20}
Four injections in 36 hr	1	1·50	1·74	0·86 {0·39-1·94}
	2	1·95	1·38	1·41 (0·93-2·13}
	3	3·31	3·40	0·97 {0·66-1·44}
Combined data: weighted mean ratio 1·12 {0·86-1·46}				

Relative activity of oestrone in distilled water and in 1 *per cent. aqueous egg albumin by a four-injection technique.*—Table 1 shows the results of three tests comparing the relative activity of oestrone in distilled water given by four injections in 36 hr and in 1 per cent. aqueous egg albumin by a similar technique. No difference in activity was observed between the two solvents, indicating that the protein exerted no effect under these conditions.

Figure 6 summarizes the results presented in this section together with the earlier results of Biggers (1951). It is seen that optimum activity is approached either by dividing the dose or by using egg albumin solution as a solvent. However, the two methods converge and when optimum conditions are reached they do not potentiate each other.

(*f*) *Relative Activities of Oestrogens under Optimum Conditions.*

In view of the results presented above, the relative activities of oestradiol-3,17β, oestriol, and diethylstilboestrol in terms of oestrone were determined.

The oestrogens were administered in distilled water by four injections in 36 hr, and for comparison the same technique was applied with 1 per cent. aqueous egg albumin. In addition, the activities of equilin and equilenin were determined in egg albumin solution only. The results have been expressed as

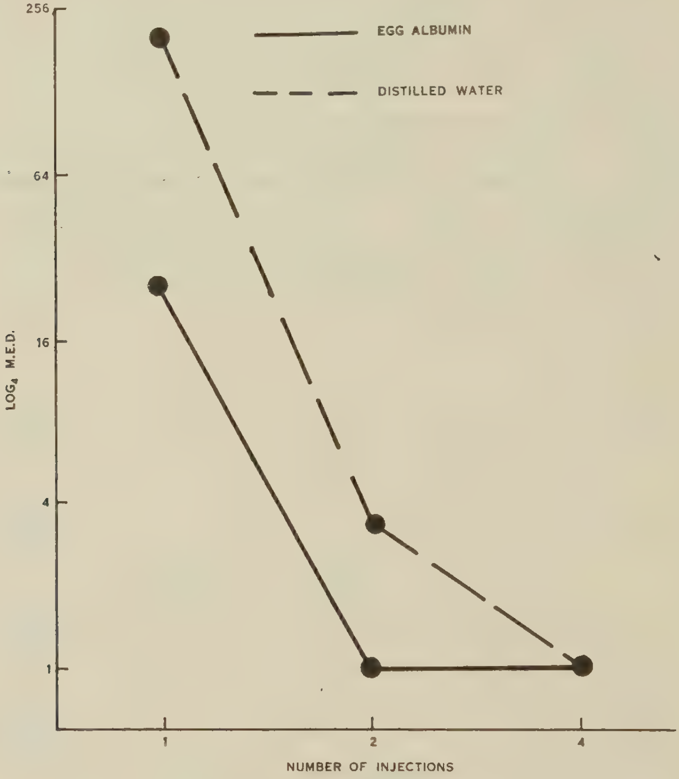

Fig. 6.—Effect of a single injection, two injections in 24 hr, and four injections in 36 hr on the response of mice to oestrone, using distilled water and 1 per cent. aqueous egg albumin as solvents.

M.E.D. ratios (see Biggers 1951) since relative potency estimates are invalid where the slopes of the dose response lines are not parallel. This was demonstrated with oestrone and oestradiol-3,17β by Biggers and Claringbold (1953) and by Claringbold (1953), and with oestrone and oestriol in the present work. The M.E.D. and its variance have been computed for each line, using individual estimates of slope instead of a pooled estimate, since earlier work (Biggers 1953*a*) and the present work have shown pooled estimates to be sometimes heterogeneous. Thus the relative activities have been calculated assuming the worst conditions and therefore any differences observed will be highly significant. The results are shown in Table 2.

Oestradiol-3,17β under all conditions of administration (Table 2) is significantly more active than oestrone (approximately 1.5 times as active), while diethylstilboestrol has the same activity as oestrone. Oestriol, however, behaves differently according to the vehicle of administration. In the presence of egg albumin oestriol has the same activity as oestrone while in distilled water its activity is considerably reduced; mere subdivision of the dose does not attain maximum activity. In this respect oestriol differs from the other oestrogens studied. It is possible that the different behaviour of oestriol is related to its water solubility. Table 3 shows the solubilities in water of various oestrogens, where it is seen that oestriol is considerably more soluble. The high solubility may favour its loss into the blood stream, and mere subdivision of the dose may fail to overcome this loss. The egg albumin, however, may hold the oestriol in the vagina and allow it to exert its maximal effect.

TABLE 2

RELATIVE ACTIVITIES OF OESTRADIOL-3,17β, OESTRIOL, DIETHYLSTILBOESTROL, EQUILIN, AND EQUILENIN IN COMPARISON WITH OESTRONE UNDER VARIOUS CONDITIONS OF ADMINISTRATION

	Present Work (optimal)			Earlier Work*
	Weighted M.E.D. Ratio and Fiducial Limits ($P = 0{\cdot}05$)	No. of Tests	No. of Animals	
Distilled water				
Oestradiol-3,17β	1·37 {1·04-1·81}	4	504	1·52 {1·18-2·02}†
Oestriol	0·09 {0·04-0·21}	1	108	0·46 {0·32-0·62}†
Diethylstilboestrol	0·76 {0·51-1·39}	2	380	—
1% Egg albumin				
Oestradiol-3,17β	1·57 {1·03-2·37}	4	520	1·75 {1·15-2·66}‡
Oestriol	1·13 {0·72-1·76}	3	412	1·09 {0·70-1·70}‡
Diethylstilboestrol	0·82 {0·57-1·19}	3	374	—
Equilin	0·84 {0·60-1·20}	1	180	—
Equilenin	0·65 {0·37-1·15}	1	180	—

* Relative potencies and their fiducial limits ($P = 0{\cdot}05$).

† Two-injection technique over 24 hr. Vehicle: 50 per cent. aqueous glycerol (Emmens 1950).

‡ Two-injection technique over 24 hr. Vehicle: 1 per cent. aqueous egg albumin (Biggers 1951).

TABLE 3

SOLUBILITIES OF OESTROGENS IN WATER

Oestrogen	Solubility (μg/ml)	References
Oestrone	1·6	Jewitt, quoted by Heard (1949)
	2·1	Butenandt and Westphal (1934)
Oestradiol-3,17β	2·7	Bischoff and Pilhorn (1948)
Oestriol	27·0	Jewitt, quoted by Heard (1949)

V. Discussion

(*a*) *The Quantitative Study of Oestrogen Activity*

The optimum conditions for the intravaginal administration of oestrone, oestradiol-3,17β, oestriol, and diethylstilboestrol are attained by two or four injections in 36 hr, using 1 per cent. aqueous egg albumin as solvent. Except with oestriol these conditions may be closely approximated by four injections over 36 hr, using distilled water as solvent. The use of four unequal injections in an increasing series provides the best approximation to the optimum with oestrone.

The greatly increased sensitivity claimed by Sulman (1952), who used a five-injection technique, has not been substantiated. His comparisons with the results of Emmens (1950*a*) are invalid for three reasons:

(1) Sulman's criterion of positivity—being positive when " . . . the number of leucocytes does not exceed the number of epithelial cells"—is highly subjective and obviously more sensitive than the criterion of cornified and nucleated cells in the absence of mucin and leucocytes used by Emmens;

(2) Emmens' figures are median effective doses while Sulman's figures are minimal effective doses; and

(3) Responses of the order quoted by Sulman are by no means exceptional in certain individual animals in our colonies; however, the median effective dose is a far more efficient statistic than the minimal effective dose in describing the sensitivity to oestrogen.

In their study of the subcutaneous technique Marrian and Parkes (1929), although using crude extracts, found the optimum conditions to be many injections (up to 20 over 24-36 hr). Thus while the two routes resemble each other with respect to time interval they are quite dissimilar with respect to subdivision of dose.

The results demonstrate the extent to which quantitative studies on the relative activity of oestrogens may be influenced by technique. Studies using the subcutaneous route are quite unreliable as indicators of relative activity at the cellular level, the results being severely biased by the effects of passage through the body, involving such factors as differential absorption and blood transport, and differential excretion and transformation to other forms. Recent studies (Biggers, Claringbold, and Emmens 1954) showed no evidence of a correlation between the sensitivity of the vagina to local and systemic administration. From the clinical point of view, information obtained by systemic administration is valuable, especially if tested on the species concerned. The recent assays of oestrogens in women by Bishop (1950) are examples of this. The nearest approach to *in vivo* assays on the cells responding is the intravaginal method, and here variations of technique can severely affect results. This is well illustrated by the low activity of oestriol in distilled water and high activity in egg albumin solution. A comparison of relative activities would seem to be meaningful only under optimum conditions of administration, and unless these are attained the results cannot readily be appraised. It seems almost certain that similar situations exist in the study of other hormones and pharmacological substances.

(*b*) *Local Action of Oestrogens*

(i) *Probability Model.*—In this work we are concerned with the probability of response and *not* with the degree of response of any one individual. For simplicity we can consider dose as a constant, e.g. the M.E.D. at the optimum conditions. The probability model then becomes a bivariate distribution with its peak at four injections and 36 hr. This means that, given the optimum conditions of administration, the probability of response is maximal.

(ii) *Uptake by the Epithelium.*—All the results presented indicate that an eight-injection technique is less effective than the four-injection technique. The smaller aliquots of oestrogen in the former are insufficient to stimulate the cellular processes, whereas the larger aliquots in the latter are more likely to produce the response, although in both instances the total dose is the same. It is necessary, therefore, to postulate a minimum dose of oestrogen which, following uptake by the epithelium, will stimulate a response.

The relative ineffectiveness of two injections in comparison with four injections is also of importance in that still larger aliquots are less effective. The larger quantity of oestrogen seems to saturate the epithelium and also leave an excess, which may be lost to the blood stream and from the vagina.

The work with 1 per cent. aqueous egg albumin supports the above theory fully. Using this solvent optimum conditions are obtained over a wider range of treatments than with distilled water. The egg albumin binds with the oestrogen and holds the hormone near its site of action, and there is thus a competition between the cells of the epithelium and the protein. Strong evidence for this view is furnished by the earlier observations of Biggers (1953*b*) with bovine plasma albumin as solvent. This protein in 0.1 per cent. solution binds to such an extent that the response is highly variable while in 0.01 per cent. solution the binding is still sufficient to increase variability. With the weakly binding egg albumin molecule no increase in variability is observed, suggesting that the epithelium may absorb the oestrogen from the protein as it is utilized with little loss into the blood stream.

(iii) *The Problem of Structure and Function.*—A large number of substances possessing oestrogenic activity are now known, and several theories as to the cause of this activity have been put forward (see Jacques 1949 for a full review of this subject). Most of these theories are based upon misleading evidence obtained from poorly conducted subcutaneous tests. The work of Emmens (1941, 1942) indicated two major groups of oestrogenic molecules—the *true oestrogens* and the *pro-oestrogens,* the latter acquiring their activity following transformation in the body to a true oestrogen. A pro-oestrogen is best defined as a substance which may be transformed into a true oestrogenic molecule, this transformation taking place at a site other than that of local action. Some of the theories, namely those based on active groups and spatial molecular configurations, completely fail to take account of this distinction, with the result that specific groups or physical chemical measurements are correlated with both pro-oestrogenic and true oestrogenic activity. It seems that in order to clarify the field much of the earlier work should be repeated to identify those substances which are true oestrogens. These substances should then be studied at a cellular level with techniques which will ensure optimum activity.

In all the above work oestradiol-3,17β stands apart from the others and it is also the most active substance at optimum conditions, all others being of the same lower activity. Although no direct evidence can be produced to show that oestradiol-3,17β is the substance to which the others are converted in the epithelium such a process seems a distinct possibility. Evidence for the intracellular conversion of oestrogens is provided by the work of Biggers and Claringbold (1953) and Claringbold (1953) on the effect of thyroid status and cyanide administration on oestrogenic activity. Much work needs to be done before any definite statements on this problem can be made.

VI. Acknowledgments

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APPENDIX I

Design, results, and analyses of the factorial experiments

To facilitate description of the factorial experiments the following conventions have been adopted:

(1) Since the levels of the independent variables were in equal steps on some scale (usually logarithmic) simple coding has been used, and is indicated by subscripts in parentheses.

(2) In each experiment the number of animals per group is constant over all treatment combinations and is indicated in the title. With the exception of Tables 9-11 the percentage response is tabulated in the appropriate cell of the design. In Tables 9-11, however, where the dosage varies in each cell, a matrix of doses is given, and this matrix is in one-to-one correspondence with the matrix of responses shown alongside.

(3) Where parallelogram designs have been employed (Tables 6-11) the analysis is carried out following a linear transformation of an independent variable. The linear regression coefficient on this variable which is obtained from the analysis must be modified allowing for this transformation, and this new coefficient is indicated at the foot of the analysis of variance by b_i^*. For further details see Claringbold, Biggers, and Emmens (1953).

TABLE 4

EFFECT OF TOTAL VOLUME OF INJECTION WITH A TWO-INJECTION TECHNIQUE AND LEVEL OF DOSAGE OF OESTRONE (18 ANIMALS PER GROUP)

Total Volume (ml)	Dose (10^{-4} μg)	
	$1{\cdot}5_{(-1)}$	$6{\cdot}0_{(1)}$
$0{\cdot}005_{(\ 2)}$	22·2	72·2
$0{\cdot}01_{(-1)}$	27·8	77·8
$0{\cdot}02_{(0)}$ (normal technique)	11·1	88·9
$0{\cdot}04_{(1)}$	5·6	50·0
$0{\cdot}08_{(2)}$	0·0	22·2

TABLE 5

EFFECT OF THREE LEVELS OF OESTRONE WHEN ADMINISTERED IN TWO, FOUR, OR EIGHT INJECTIONS OVER 24 HR (18 ANIMALS PER GROUP)

	Frequency of Injection	Dose Oestrone ($10^{-4}\,\mu$g)		
		$2_{(-1)}$	$4_{(0)}$	$8_{(+1)}$
Rep. I	$2_{(-1)}$	38·9	55·6	66·7
	$4_{(0)}$	21·8	55·6	72·0
	$8_{(+1)}$	16·7	33·3	50·0
Rep. II	$2_{(-1)}$	44·4	88·9	94·4
	$4_{(0)}$	94·4	83·3	88·9
	$8_{(+1)}$	66·7	88·9	94·4
Rep. III	$2_{(-1)}$	33·3	61·1	66·7
	$4_{(0)}$	66·7	66·7	72·2
	$8_{(+1)}$	16·7	38·9	61·1

Analysis of Variance

Source of Variation	D.f.	Sum of Squares	Mean Square	*F*	*P*
Replications	(2)	(2994·9)	(1497·5)		
Linear	1		103·1	2·1	$>0\cdot05$
Quadratic	1		2891·8	59·6	$<0\cdot001$
Frequency of injection	(2)	(538·3)	(269·2)		
Linear	1		168·7	3·5	$>0\cdot05$
Quadratic	1		369·6	7·6	0·01-0·001
Doses	(2)	(1678·3)	(839·2)		
Linear	1		1636·5	33·7	$<0\cdot001$
Quadratic	1		41·8	0·8	$>0\cdot05$
Interactions	20	1136·0	56·8	1·2	$>0\cdot05$
Theoretical variance	∞		48·5		

TABLE 6

EFFECT OF TIME INTERVAL (X_1), DOSE OF OESTRONE (X_2), AND FREQUENCY OF INJECTION (12 ANIMALS PER GROUP

Time Interval	Frequency of Injection	Dose Oestrone ($10^{-4}\mu g$)		
		$2{\cdot}6_{(0{\cdot}415)}$	$5{\cdot}3_{(0{\cdot}585)}$	$10{\cdot}5_{(1{\cdot}585)}$
$16_{(-1)}$	$2_{(-1)}$	25·0	50·0	75·0
	$4_{(0)}$	41·7	66·7	91·7
	$8_{(1)}$	33·3	58·3	66·7
		$1{\cdot}8_{(-1)}$	$3{\cdot}5_{(0)}$	$7{\cdot}0_{(1)}$
$24_{(0)}$	2	8·3	50·0	66·7
	4	33·0	58·3	91·7
	8	25·0	50·0	83·3
		$1{\cdot}2_{(-1{\cdot}585)}$	$2{\cdot}3_{(-0{\cdot}585)}$	$4{\cdot}7_{(0{\cdot}415)}$
$36_{(1)}$	2	41·7	58·3	66·7
	4	50·0	83·3	83·3
	8	58·3	58·3	83·3

Analysis of Variance

Transformation: $X_2 = X_2{}^* + 0{\cdot}585\ X_1$

Source of Variation	D.f.	Sum of Squares	Mean Square	F	P
Doses	(2)	(3368·4)	(1684·2)		
Linear	1		3362·0	44·6	<0·001
Quadratic	1		6·4	0·2	>0·05
Frequency	(2)	(637·3)	(318·7)		
Linear	1		138·0	1·8	>0·05
Quadratic	1		499·3	6·6	0·05-0·01
Time Interval (X_1)	(2)	(287·5)	(143·8)		
Linear	1		107·3	1·4	>0·05
Quadratic	1		180·2	2·4	>0·05
Interactions	20	629·0	31·5	0·4	0·05-0·01
Theoretical variance	∞		75·3		

Significance of linear regression coefficient of X_1: $b_1{}^* = 10{\cdot}44$, $s_{b1}{}^* = 2{\cdot}37$; $t_{[\infty]} = 4{\cdot}40$, $P<0{\cdot}001$.

TABLE 7

EFFECT OF TIME INTERVAL (X_1), DOSE OF OESTRADIOL-3,17β (X_2), AND FREQUENCY OF INJECTION (12 ANIMALS PER GROUP)

Time Interval (hr)	Frequency of Injection	Dose Oestradiol-3,17β (10^{-4} μg)		
		$2{\cdot}6_{(0{\cdot}415)}$	$5{\cdot}3_{(0{\cdot}585)}$	$10{\cdot}5_{(1{\cdot}585)}$
$16_{(-1)}$	$2_{(-1)}$	50·0	66·7	75·0
	$4_{(0)}$	83·3	100·0	100·0
	$8_{(1)}$	58·3	66·7	83·3
		$1{\cdot}8_{(-1)}$	$3{\cdot}5_{(0)}$	$7{\cdot}0_{(1)}$
$24_{(0)}$	2	50·0	75·0	75·0
	4	66·7	66·7	100·0
	8	41·7	75·0	83·3
		$1{\cdot}2_{(-1{\cdot}585)}$	$2{\cdot}3_{(-0{\cdot}585)}$	$4{\cdot}7_{(0{\cdot}415)}$
$36_{(1)}$	2	16·7	41·7	58·3
	4	33·3	91·7	83·3
	8	41·7	50·0	83·3

Analysis of Variance

Transformation: $X_2{}^* = X_2 + 0{\cdot}585\ X_1$

Source of Variation	D.f.	Sum of Squares	Mean Square	*F*	*P*
Doses	(2)	(2195·5)	(1097·8)		
Linear	1		2146·0	28·5	$<0{\cdot}001$
Quadratic	1		49·5	0·7	$>0{\cdot}05$
Frequency	(2)	(1414·3)	(707·2)		
Linear	1		132·0	1·8	$>0{\cdot}05$
Quadratic	1		1282·3	17·0	$<0{\cdot}001$
Time interval (X_1)	(2)	(804·8)	(402·4)		
Linear	1		796·5	10·6	0·05-0·01
Quadratic	1		38·3	0·5	$>0{\cdot}05$
Interactions	20	1025·3	51·3	0·7	$>0{\cdot}05$
Theoretical variance	∞		75·3		

Significance of linear regression coefficient of X_1: $b_1{}^* = -0{\cdot}27$, $s_{b1}{}^* = 2{\cdot}37$; $t_{[\infty]} = 0{\cdot}11$, $P > 0{\cdot}05$.

Table 8

EFFECT OF TIME INTERVAL (X_1), DOSE OF DIETHYLSTILBOESTROL (X_2), AND FREQUENCY OF INJECTION (12 ANIMALS PER GROUP)

Time Interval (hr)	Frequency of Injection	Dose Diethylstilboestrol (10^{-4} µg)		
		$6\cdot0_{(-0\cdot415)}$	$12\cdot0_{(0\cdot585)}$	$24\cdot0_{(1\cdot585)}$
$16_{(-1)}$	$2_{(-1)}$	75·0	58·3	100·0
	$4_{(0)}$	66·7	83·3	91·7
	$8_{(1)}$	41·7	58·3	66·7
		$4\cdot0_{(-1)}$	$8\cdot0_{(0)}$	$16\cdot0_{(1)}$
$24_{(0)}$	2	50·0	100·0	83·3
	4	75·0	83·3	100·0
	8	25·0	58·3	66·7
		$2\cdot7_{(-1\cdot585)}$	$5\cdot3_{(-0\cdot585)}$	$10\cdot7_{(0\cdot415)}$
$36_{(1)}$	2	33·3	50·0	75·0
	4	33·3	66·7	91·7
	8	8·3	50·0	75·0

Analysis of Variance

Transformation: $X_2^* = X_2 + 0\cdot585\ X_1$

Source of Variation	D.f.	Sum of Squares	Mean Square	F	P
Doses	(2)	(2911·7)	(1455·9)		
Linear	1		2892·1	38·4	$<0\cdot001$
Quadratic	1		19·6	0·3	$>0\cdot05$
Frequency	(2)	(1616·4)	(808·2)		
Linear	1		844·1	11·2	$<0\cdot001$
Quadratic	1		772·3	10·3	0·01-0·001
Time interval (X_1)	(2)	(851·9)	(421·0)		
Linear	1		611·3	8·1	0·01-0·001
Quadratic	1		240·6	3·2	0·1-0·05
Interaction	20	1351·4	67·57	0·9	$>0\cdot05$
Theoretical variance	∞		75·3		

Significance of linear regression coefficient of X_1: $b_1^* = 1\cdot59$, $s_{b_1}^* = 2\cdot37$; $t_{[\infty]} = 0\cdot67$; $P>0\cdot05$.

TABLE 9

EFFECT OF FREQUENCY OF INJECTION IN 24 HR (X_3) AND DOSE OF OESTRIOL (X_2) (18 ANIMALS PER GROUP)

Frequency of Injection	Dose Oestriol (10^{-4} μg)			Response		
$2_{(-1)}$	$128_{(2)}$	$256_{(3)}$	$512_{(4)}$	33·3	55·6	66·7
$4_{(0)}$	$16_{(-1)}$	$32_{(0)}$	$64_{(1)}$	50·0	83·3	88·9
$8_{(1)}$	$2_{(-4)}$	$4_{(-3)}$	$8_{(-2)}$	0·0	11·1	11·1

Analysis of Variance

Transformation: $X_2^* = X_2 + 3X_3$

Source of Variation	D.f.	Sum of Squares	Mean Square	F	P
Doses	(2)	(625·2)	(312·6)		
Linear	1		555·8	11·5	<0·001
Quadratic	1		69·4	1·4	>0·05
Frequency (X_3)	(2)	(3205·6)	(1602·8)		
Linear	1		1427·0	29·4	<0·001
Quadratic	1		1778·6	36·7	<0·001
Interactions	4	49·52	12·38	0·3	>0·05
Theoretical variance	∞		48·5		

Significance of linear regression coefficient of X_3: $b_3^* = 13 \cdot 45$, $s_{b3}^* = 8 \cdot 99$; $t_{[\infty]} = 1 \cdot 50$, $P > 0 \cdot 05$.

TABLE 10

EFFECT OF TIME INTERVAL (X_1) AND DOSE OF OESTRONE (X_2) (20 ANIMALS PER GROUP)

Time Interval (hr)	Dose Oestrone (10^{-4} μg)				Response			
$0 \cdot 9_{(-3)}$	$64_{(3)}$	$128_{(5)}$	$256_{(7)}$	$512_{(9)}$	25	30	60	85
$2 \cdot 7_{(-1)}$	$16_{(-1)}$	$32_{(1)}$	$64_{(3)}$	$128_{(5)}$	30	55	60	70
$8 \cdot 0_{(1)}$	$4_{(-5)}$	$8_{(-3)}$	$16_{(-1)}$	$32_{(1)}$	15	30	35	60
$24 \cdot 0_{(3)}$	$1_{(-9)}$	$2_{(-7)}$	$4_{(-3)}$	$8_{(-3)}$	20	45	50	70

Analysis of Variance

Transformation: $X_2^* = X_2 + 2X_1$

Source of Variation	D.f.	Sum of Squares	Mean Square	F	P
Doses	(3)	(1949·0)	(649·7)		
Linear	1		1922·7	46·9	<0·001
Quadratic	1		3·8	0·3	>0·05
Cubic	1		22·5	0·5	>0·05
Time interval	3	260·3	86·8	2·0	>0·05
Interactions	9	202·5	22·5	0·5	>0·05
Theoretical variance	∞		43·3		

Significance of linear regression coefficient of X_1: $b_1^* = 17{\cdot}74$, $s_b^* = 3{\cdot}29$; $t_{[\infty]} = 5{\cdot}38$; $P<0{\cdot}001$.

TABLE 11

EFFECT OF TIME INTERVAL (X_1) AND OESTROGEN (X_2) (12 ANIMALS PER GROUP)

Oestrogen	Time Interval	Dose ($10^{-4}\,\mu$g)			Response		
Oestrone	$13{\cdot}5_{(-1)}$	$6_{(0)}$	$12_{(1)}$	$24_{(2)}$	47·7	50·0	66·7
	$18{\cdot}0_{(0)}$	$3_{(-1)}$	$6_{(0)}$	$12_{(1)}$	50·0	58·3	83·3
	$24{\cdot}0_{(1)}$	$1{\cdot}5_{(-2)}$	$3_{(-1)}$	$6_{(0)}$	41·7	33·3	83·3
Oestradiol-3,17β	13·5	$4_{(0)}$	$8_{(1)}$	$16_{(2)}$	66·7	58·3	75·0
	18·0	$2_{(-1)}$	$4_{(0)}$	$8_{(1)}$	75·0	91·7	100·0
	24·0	$1_{(-2)}$	$2_{(-1)}$	$4_{(0)}$	33·3	75·0	66·7
Diethylstilboestrol	13·5	$18_{(0)}$	$36_{(1)}$	$72_{(2)}$	41·7	33·3	66·7
	18·0	$9_{(-1)}$	$18_{(0)}$	$36_{(1)}$	50·0	50·0	91·7
	24·0	$4{\cdot}5_{(-2)}$	$9_{(-1)}$	$18_{(0)}$	33·3	83·3	100·0

Analysis of Variance

Although all parts of the experiment above were performed simultaneously the analysis has been conducted in three parts for simplicity. Transformation: $X_2^* = X_2 + X_1$

Source of Variation	D.f.	Sum of Squares	Mean Square	F	P
A. Oestrone					
Doses	(2)	(771·6)	(385·8)		
Linear	1		621·8	8·3	0·01-0·001
Quadratic	1		149·8	2·0	>0·05
Time interval (X_1)	2	89·2	44·6	0·6	>0·05
Interactions	4	118·3	29·6	0·4	>0·05

Significance of linear regression coefficient of X_1: $b_1^* = 10{\cdot}41$, $s_{b1}^* = 5{\cdot}02$; $t_{[\infty]} = 2{\cdot}07$; $0{\cdot}05 > P > 0{\cdot}02$.

TABLE 11 (*Continued*)

Source of Variation	D.f.	Sum of Squares	Mean Square	F	P
B. Oestradiol-3,17β					
Doses	(2)	(381·1)	(190·6)		
Linear	1		359·8	4·8	0·05-0·01
Quadratic	1		21·3	0·4	>0·05
Time interval (X_1)	(2)	(774·8)	(387·4)		
Linear	1		35·2	0·5	>0·05
Quadratic	1		739·6	9·8	0·01-0·001
Interactions	4	250·9	62·7	0·8	>0·05

Significance of linear regression coefficient of X_1: $b_1{}^* = 5 \cdot 32$, $s_{b_1}{}^* = 5 \cdot 02$; $t_{[\infty]} = 1 \cdot 05$; $P > 0 \cdot 05$.

C. Diethylstilboestrol					
Doses	(2)	(1407·9)	(704·0)		
Linear	1		1327·9	17·6	<0·001
Quadratic	1		80·0	1·1	>0·05
Time interval (X_1)	(2)	(471·2)	(235·6)		
Linear	1		461·0	6·1	0·05-0·01
Quadratic	1		10·2	0·1	>0·05
Interactions	4	446·2	111·5	1·5	>0·05

Significance of linear regression coefficient of X_1: $b_1{}^* = 23 \cdot 64$, $s_{b_1}{}^* = 5 \cdot 02$; $t_{[\infty]} = 4 \cdot 71$; $P < 0 \cdot 001$. Theoretical variance 75·3, D.f. ∞.

TABLE 12

EFFECT OF TIME INTERVAL, VEHICLE OF ADMINISTRATION, AND DOSE OF OESTRONE (18 ANIMALS PER GROUP)

Vehicle	Time Interval (hr)	Dose of Oestrone (10^{-4} μg)	
		$1 \cdot 4_{(-1)}$	$5 \cdot 6_{(1)}$
1·0% Egg albumin	$26_{(-1)}$	66·7	94·4
	$36_{(0)}$	72·2	94·4
	$49_{(1)}$	55·6	100·0
Distilled water	26	33·3	66·7
	36	66·7	100·0
	49	38·9	72·2

Analysis of Variance

Source of Variation	D.f.	Sum of Squares	Mean Square	*F*	*P*
Vehicle	1	435·0	435·0	9·0	0·01-0·001
Time interval	(2)	(387·8)	(193·9)		
Linear	1		6·5	0·1	$>0·05$
Quadratic	1		381·4	7·9	0·01-0·001
Doses	1	1686·7	1686·7	34·8	$<0·001$
Dose × time interval	(2)	(290·4)	(145·2)		
Departures from parallelism	1		5·1	0·1	$>0·05$
Opposed curvature	1		285·3	5·9	0·05-0·01
Other interactions	5	110·3	22·1	0·5	$>0·05$
Theoretical variance	∞		48·5		

TABLE 13

EFFECT OF FOUR INJECTIONS IN 36 HR WITH UNEQUALLY DIVIDED DOSES, OESTRONE BEING ADMINISTERED IN DISTILLED WATER

A. Design

Groups	Dose Oestrone per Injection (10^{-4} μg)				Total Dose
	1st	2nd	3rd	4th	
−2	4	2·67	1·33	0	$8_{(+3)}$*
−1	3	2·33	1·67	1	8
0	2	2	2	2	8
1	1	1·67	2·33	3	8
2	0	1·33	2·67	4	8

* Four levels of total dose were used, i.e., 1, 2, 4, and 8 × 10^{-4} μg, coded −3, −1, 1, 3. Only the doses for the highest group are shown above, the others being obtained by successive division by 2.

B. Results (18 animals per group)

Group	Total Dose ($\times 10^{-4}$ μg)			
	$1_{(-3)}$	$2_{(-1)}$	$4_{(1)}$	$8_{(3)}$
−2	0	16·7	33·3	50·0
−1	5·6	33·3	33·3	61·1
0	16·7	38·9	61·1	77·8
1	11·1	50·0	55·6	88·9
2	5·6	38·9	55·6	55·6

SOME VARIABLES AFFECTING THE USE OF COWPEA AS AN ASSAY HOST FOR CUCUMBER MOSAIC VIRUS

By N. C. Crowley*

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Summary

A study was made of the factors affecting the production of local lesions by cucumber mosaic virus on cowpea (*Vigna sinensis* (L.) Endl. ex Hassk.).

The following facts have been established:

Cowpea is a satisfactory host for quantitative work with cucumber mosaic virus at dilutions of inoculum from 1/20 to 1/200. The half-leaf technique can be used to advantage.

Cucumber mosaic virus inoculum is most infectious at pH 8, has greatest longevity at pH 7, and will tolerate a pH range from 4 to 10.

Alauxite and carborundum (both 500-mesh) were superior to all other abrasives tested. Washing of inoculated leaves did not increase the number of lesions developed.

More lesions developed on cowpea plants placed under fluorescent lights at 200 f.c. than on plants placed either in the glass-house or under higher light intensities.

The rapid drying of inoculated leaves increased the number of lesions developed.

The use of leaf-culture techniques has not made possible a reduction in the variation between plants.

I. Introduction

Sill and Walker (1952) recently published a description of the technique they developed using cowpea (*Vigna sinensis* (L.) Endl. ex Hassk.) as an assay host for cucumber mosaic virus and reviewed the relevant literature. In virus investigations at Waite Institute a similar technique had been developed independently. In the main our findings have confirmed those of Sill and Walker but additional factors which are of importance in quantitative work have been investigated and are published as an extension of Sill and Walker's work.

II. Materials and Methods

The strain of cucumber mosaic virus used was obtained from Mr. L. L. Stubbs, of the Department of Agriculture, Victoria, and had been isolated by him from naturally infected spinach. Inoculum for all experimental work was extracted from 3-weeks-old cucumber seedlings within 10 days of their being inoculated.

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All cowpea seedlings used were of the variety Blackeye from seed of Californian origin which was obtained through Mr. W. Hartley, of the Division of Plant Industry, C.S.I.R.O., Canberra.

All cowpea leaves were inoculated by means of two strokes towards the tip with a ground-glass spatula, as this was found to result in the production of more lesions than other methods. This is not in agreement with the findings of Sill and Walker (1952), who used a circular stroking method with a glass spatula on all leaves, but the most successful inoculation technique may be expected to vary with different workers. Plants used were raised individually in small pots of John Innes compost, placed in semi-darkness in the laboratory when 13-15 days old, inoculated the following day, and placed in an artificially illuminated growth chamber immediately after inoculation.

Experiments involving only four treatments were carried out by the half-leaf method, using 16 or 20 replicates of each treatment in a randomized block design so that each treatment occurred on every plant, and occurred an equal number of times in each leaf position. Where larger numbers of treatments were necessary, experiments were carried out either in an incomplete block design, or by whole-leaf comparisons where it was imperative that no run-over of inoculum should occur.

III. Experimental

The work of Sill and Walker (1952) has been independently confirmed in the following particulars:

(1) The variety Blackeye is one of the most susceptible varieties of cowpea to cucumber mosaic virus;

(2) Cowpea seedlings are most susceptible when 14-16 days old;

(3) Their susceptibility is greatly increased by keeping the plants in darkness for 24 hr prior to inoculation;

(4) Tobacco (*Nicotiana tabacum*) is a more infective source of cucumber mosaic virus than either *N. glutinosa* or cucumber (*Cucumis sativa*). Additional data have been obtained from the present work and are discussed in detail below.

(*a*) *Effect of Dilution*

In quantitative work with plant viruses using the local lesion technique, it was shown by Best (1935), working with tobacco mosaic and tomato spotted wilt viruses, that the numbers of local lesions produced are directly proportional to virus concentration only over a limited portion of the dilution range; i.e. only over the "straight line portion where a change in relative concentration is accompanied by an equivalent change in resultant lesions." No dilution curve for cucumber mosaic virus has been published although a knowledge of the effect of dilution is essential for all quantitative work. Hence an experiment was carried out using 24 replicates of each dilution in an incomplete block design covering the range from undiluted infective sap to a dilution of 1 in 5120. The results are graphed in Figure 1.

These results were verified by repeated experiments. The sharp rise in infectivity on first diluting infective sap is probably due to several factors; firstly, buffering is known to increase the infectivity of this virus; secondly, dilution would result in the dispersion of virus aggregates and thus result in a higher concentration of infective units; and thirdly, dilution would also result in the dilution of toxic constituents of plant sap which inhibit virus infection.

The steeply sloping portion of the curve occurs over the dilution range from 1/10 to approximately 1/150. At lower dilutions, 1/5-1/10, and at all dilutions greater than 1/200 only small changes in lesion numbers result from large changes in dilution.

In quantitative work with cucumber mosaic virus at Waite Institute, a dilution of 1/50 is generally used, being near the centre of the straight-line portion of the curve, and producing lesions in numbers sufficiently large for accurate counting. It is, however, preferable where possible to use several dilutions.

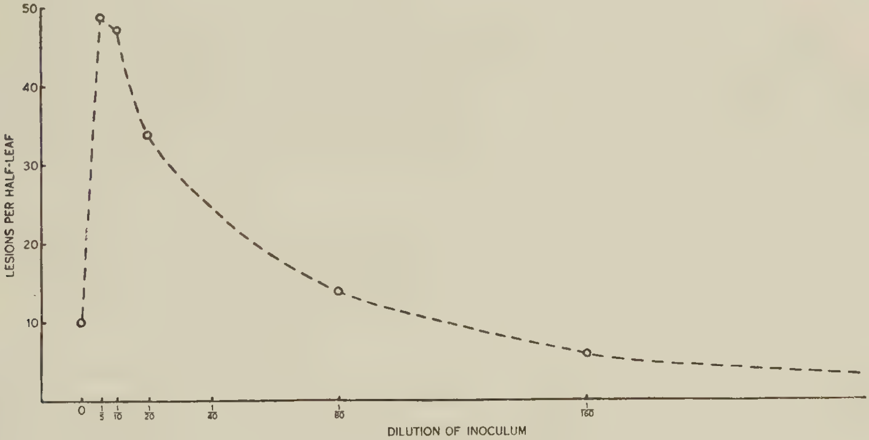

Fig. 1.—Effect of dilution of inoculum on lesions produced by cucumber mosaic virus on cowpea.

(*b*) *The Half-leaf Method*

The use of the half-leaf technique on a plant such as cowpea, which has small, easily wettable leaves, incurs the risk of error from inoculum running across from one half-leaf to the other during the inoculation procedure, and for this reason Sill and Walker (1952) used whole-leaf comparisons in their investigations. As the half-leaf method allows the comparison of four treatments on every plant, thus increasing the experimental accuracy by spreading the effects of the between-plant differences on double the number of treatments, and frequently making possible the elimination of these differences in the analysis of the results, it was thought that the advantages of using this technique might outweigh the disadvantages. Experiments were devised to determine the magnitude of the error due to run-over of inoculum. Table 1 shows the results of experiments in which lots of 20 plants were inoculated on one half-leaf with a

1/40 dilution of infective cucumber sap and on the other half-leaf with distilled water.

TABLE 1

AMOUNT OF RUN-OVER OF INOCULUM USING THE HALF-LEAF METHOD

Inoculum Used	Average Lesions per Half-leaf				
	Experiment				
	1	2	3	4	Mean
Infective inoculum	61	80	146	103	98
Distilled water	0·4	0·1	0·3	0·2	0·2

Table 2 shows the results of an experiment to determine whether there was any significant difference in the number of local lesions produced by a weak inoculum, firstly when the same inoculum was used on the other half of each leaf, and secondly when a strong inoculum was used on the other half of each leaf.

TABLE 2

EFFECT OF RUN-OVER OF A STRONG INOCULUM ON NUMBER OF LESIONS PRODUCED BY A WEAK INOCULUM ON THE SAME LEAF

Treatment		Average Lesions per Half-leaf		
		Leaf Half		
		Left	Right	Mean
Weak with weak.	Weak	2·0	2·3	2·1
Weak with strong.	Weak	2·5	2·6	2·6
	Strong	187	171	179

Differences between treatments or treatment means not significant at $P = 0{\cdot}05$.

The results of both experiments indicate that a slight "run-over" from one half-leaf to another does occur but it is not of sufficient magnitude to produce a significant difference in the results of any treatment. It is concluded that the half-leaf method is the most satisfactory for use in quantitative work with cucumber mosaic virus on cowpea, except in experiments such as dilution curves or pH-infectivity determinations where it is imperative that no "run-over" should occur.

(c) *Effect of Abrasives*

Various abrasives have been used to facilitate the mechanical inoculation of plant viruses since the first demonstration of their value by Rawlins and Thompkins (1934), who showed that several viruses which were otherwise difficult to transmit mechanically could be readily transmitted with the aid of carborundum powder. Costa (1944) first reported the use of carborundum powder in the inoculation of cowpea var. Black with cucumber mosaic virus. He also showed marked differences to exist in the effectiveness of different grades of carborundum and alauxite in promoting infection of a tobacco hybrid by tobacco mosaic virus.

As not all of the abrasives used by Costa were available in Australia, and because of the recent tendency by some virus workers to use celite in preference to carborundum (Kalmus and Kassanis 1945; Hutton and Peak 1951) several of the available abrasives were tested to determine which was the most efficient for quantitative work with cucumber mosaic virus on cowpea. The results are set out in Table 3.

TABLE 3

COMPARISON OF EFFICIENCY OF ABRASIVES IN INOCULATING COWPEAS

Abrasive	Average Number of Lesions per Half-leaf				
	Experiment				
	1	2	3	4	Mean
None	7	109	25	7	37
Increase over controls with					
Carborundum 3F	39***	168***	60***	97***	91
Alauxite 400	55***	175***	57***	61***	87
Hyflo Super Cel	24***	41*	21*	35***	30

* Significant at $P = 0{\cdot}05$.
*** Significant at $P = 0{\cdot}001$.

Earlier experiments had shown 3F carborundum and 400 alauxite to be superior to any coarser grades of either abrasives though the finer (500-mesh) grades of both are slightly superior. Carborundum and alauxite are clearly most efficient inoculation aids and both resulted in up to a tenfold increase in the number of lesions developed; both were far superior to celite (Hyflo Super Cel).

(d) *Washing off the Inoculum*

Since the first report of Holmes (1929) that the washing of inoculated leaves with a jet of water immediately after inoculation increases, rather than decreases the number of lesions produced, workers have adopted the technique of washing inoculated leaves shortly after inoculation. Samuel and Bald (1933) showed that washing off the inoculum immediately after inoculation resulted in a large increase in local lesions developed when undiluted infective sap was used as inoculum, but only a slight increase when dilute buffered inoculum was used. They support the suggestion of Holmes that washing off removes some plant constituents in the inoculum which have toxic effects on the slightly damaged cells of the inoculated leaf. In dilute inoculum the constituents would be correspondingly diluted before inoculation, and thus less effective.

TABLE 4

EFFECT OF WASHING INOCULATED LEAVES AFTER VARIOUS TIME INTERVALS

Time Interval	Average Lesions per Half-leaf				
	Experiment				
	1	2	3	4	Mean
Immediately	51	170	57	64	85
30 Sec	67**	192	64	85*	102
5 Min	76**	223**	76**	79	114
Unwashed	56	211*	72**	79	105

* Significant at $P = 0{\cdot}05$.
** Significant at $P = 0{\cdot}01$.

A series of experiments was carried out to determine the importance of this factor in local lesion production by cucumber mosaic virus on cowpea. The results tabulated in Table 4 indicate that this factor is not of critical importance. Variations of up to 30 min in the interval between inoculations and washing off produced small differences in the number of lesions developed. It is concluded that in quantitative work washing off should not be carried out as it adds another variable to all experiments and enhances the risk of "run-over" from one half-leaf to another. The lack of agreement between these results and those of previous workers is attributed to the use of dilute buffered inoculum (1/50) in which any toxic constituent would be correspondingly diluted before inoculation.

(*e*) *Effect of pH on Infectivity of Inoculum*

Published data on the effect of pH on the infectivity of cucumber mosaic virus appear conflicting. Stanley (1935) reported that "cucumber mosaic is inactivated as the pH is increased or decreased from 5.7." Nienow (1948) reported the optimum pH for the inoculation of cowpea leaves to be 6.5. Bearing indirectly on this Uppal (1934) reported buffers of pH 6.6-7.2 to be the most effective in eluting the virus.

As these reports are thought to be rather variable investigations were carried out to determine at what pH inoculum would induce most lesions on cowpea. The effect measured is undoubtedly one affecting both the plant and the virus, but for the purpose of these investigations it is information on this interaction which is sought.

Inoculum buffered in composite buffer (Best and Samuel 1936) at pH 8 consistently produced significantly more lesions than inoculum buffered at either higher or lower pH.

Several experiments were carried out to compare the infectivity of cucumber mosaic virus at pH values from 2.0 to 12.0.

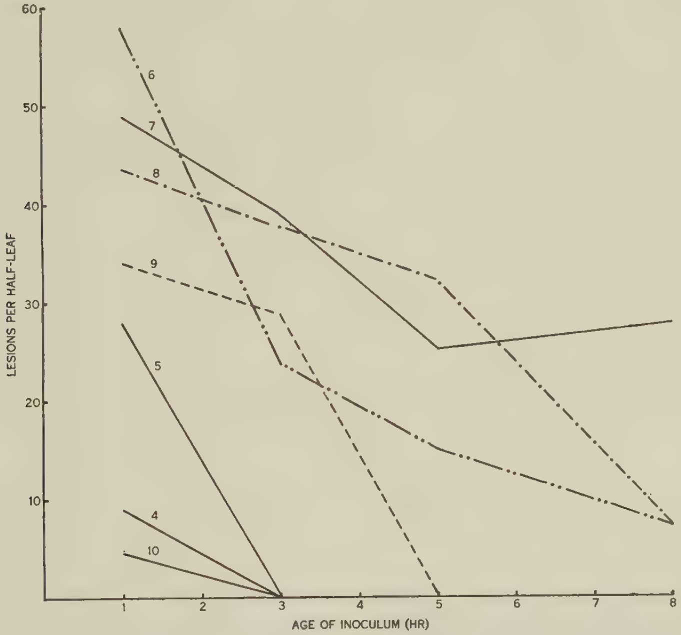

Fig. 2.—Longevity of cucumber mosaic virus *in vitro* at pH values from 4 to 10.

It was found that the values obtained varied in different experiments, depending on the period of time for which the inoculum was kept at the different pH values. It was concluded that the only satisfactory method of investigating the pH range of the virus is to determine the longevity of inoculum at different pH values. It is clear from the experimental results shown in Figure 2 that the pH range of cucumber mosaic virus varies markedly with the period the virus is exposed to that pH. Inoculum less than an hour old is infective at all pH values from 3.0 to 10.0, while inoculum 8 hr old is infective only over the pH range from 6.0 to 8.0. It follows that it is best to carry out all quantitative work with cucumber mosaic virus inoculum buffered to pH 7.0-8.0, for within this pH range the decline in infectivity over a period is least.

(*f*) *Effect of Illumination Conditions on Lesion Development*

Numerous workers have demonstrated the marked effect of light intensity on virus multiplication and on symptom development by virus-infected plants. The influence of this factor on lesion production by cowpea was investigated.

It was found at an early stage that more lesions developed on plants placed under fluorescent lights than on those replaced in the glass-house after inoculation. Illumination conditions under the artificial lights were found to affect greatly the number of lesions developed and experiments were conducted to determine the most favourable illumination conditions for lesion production. The results are tabulated in Table 5. A low light intensity of 200 f.c. has consistently resulted in the production of more lesions than an 800 f.c. intensity, while a continuous illumination at either intensity resulted in consistently more lesions than either 12 hr illumination per 24 hr or continuous darkness.

TABLE 5

EFFECT OF POST-INOCULATION LIGHT INTENSITY OF LESION PRODUCTION

Treatment	Average Lesions per Leaf				
	Experiment				
	1	2	3	4	Mean
800 f.c.	47	238	53	51	45
200 f.c.	114***	416***	80***	97***	77

*** Differences significant at $P = 0\cdot001$.

The results indicate that a light intensity as high as 800 f.c. inhibits the multiplication of cucumber mosaic virus, possibly through accumulation of products of photosynthesis, which at a lower light intensity may be produced only at a sufficient rate to supply the needs of the plant respiration at that temperature.

(*g*) *Rapid Drying of Inoculated Leaves*

Recently Yarwood (1952) reported that the number of lesions produced by tobacco mosaic virus on *N. glutinosa* could be increased more than sevenfold by rapidly drying the inoculated leaves within 10-15 sec of inoculating.

Several experiments were carried out with cucumber mosaic virus on cowpea in which one leaf of each plant inoculated was washed and dried by means of a stream of warm air within 20-30 sec of the completion of the inoculation; the other leaf of every plant was washed and allowed to dry in the ordinary way; this taking about 30 min. The results of four experiments are set out in Table 6.

Rapid drying has consistently resulted in a significantly higher lesion production by cowpeas, but the effect is not sufficient to greatly increase the sensitivity of the reaction, and in quantitative work the addition of another factor which could increase the variation between plants is most undesirable.

(*h*) *Leaf-culture Techniques*

An attempt was made to reduce the variability between cowpea plants by detaching all leaves soon after inoculation and keeping them alive in leaf culture on tap water, or on a 10 per cent. sucrose solution in petri dishes until the lesions developed were countable. The technique used was one of several described by Yarwood (1946). Using this method more lesions were produced on detached leaves in petri dishes than on leaves still attached to the plant, but the lesions produced tended to be of a more spreading nature, making accurate counting difficult. In addition, the variation in the results of lesion counts on leaves in the petri dishes was no less than the variation in lesion counts of leaves attached to the plant, indicating that the more constant post-inoculation environment was not important.

TABLE 6

EFFECT OF RAPID DRYING OF INOCULATED LEAVES ON LESION PRODUCTION

Treatment	Average Lesions per Leaf				
	Experiment				
	1	2	3	4	Mean
Dried	77***	34***	74*	77	66
Undried	58	18	53	71	50

* Significant at $P = 0{\cdot}05$.
*** Significant at $P = 0{\cdot}001$.

(*i*) *Statistical Analysis*

In studies of the frequency distribution of local lesions produced by tobacco mosaic and tomato bushy stunt viruses, Kleczkowski (1949) found both to be skew and 'leptokurtic,' the standard error increasing with the mean, and he has put forward a transformation, $y = \log_{10}(x + c)$, where x is the number of lesions and c a constant.

An experiment was carried out to determine the value of the constant c, using Kleczkowski's method. Fifty cowpeas were inoculated on all four half-leaves with a 1/50 dilution of infected cucumber sap.

Analysis of results of the lesion counts showed the regression of the standard error on the mean to be highly significant. A value of 2.6 has been calculated for the constant c, and this transformation has been used for the statistical analysis of all results.

IV. ACKNOWLEDGMENTS

The author wishes to acknowledge the helpful advice of Dr. N. T. Flentje throughout the progress of the work; the help of both Dr. C. G. Hansford and Dr. N. T. Flentje in the preparation of the manuscript; of Mrs. I. Mathison with the statistical work; and Miss Y. Cuddihy for able technical assistance.

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THE EFFECT OF MOLYBDATE ON THE ACTIVITY OF TOMATO ACID PHOSPHATASES

By D. SPENCER[*]

[*Manuscript received February* 3, 1954]

Summary

The effectiveness of sodium molybdate as an inhibitor of tomato acid phosphatases was examined. Significant inhibition of cell-free dialysed extracts was obtained with as low as 10^{-7}M sodium molybdate. A range of substrates, including a number of glycolytic intermediates, was tested and all except glucose 1-phosphate were hydrolysed by tomato leaf phosphatase preparations. Hydrolysis was always inhibited by molybdate. The inhibition was competitive; $K_i = 10^{-5}$M.

A measure of the *in vivo* phosphatase activity of intact living roots was obtained by immersing roots in a solution of *p*-nitrophenyl phosphate, and measuring the rate of formation of *p*-nitrophenol in the external solution. Molybdate inhibited phosphatases in whole root systems and in cell-free root extracts to the same degree.

From the data it is concluded that molybdate, at physiological concentrations, does inhibit the phosphatases of tomato *in vivo,* at least in tomato root cells, and that the inhibition of phosphatases is a possible metabolic function of molybdenum.

I. INTRODUCTION

It has been shown that preparations of plant acid phosphatases are inhibited by molybdate (Massart and Vermeyen 1942; Bossard 1947; Courtois and Anagnostopoulos 1949). Rothstein and Meier (1949) have shown a similar effect of molybdate on the cell surface phosphatases of yeast. Phosphatases are known to be widely distributed in plants (Ignatieff and Wasteneys 1936; Yin 1945), and to occur in a variety of tissues. They have been reported as hydrolysing a wide range of substrates, including key metabolic compounds such as hexose diphosphate, glucose 6-phosphate, and adenosine triphosphate, as well as the two common phosphatase substrates *β*-glycerophosphate and *p*-nitrophenylphosphate (PNPP).

Experiments described in this paper were designed to examine further the effect of molybdate on plant acid phosphatases, and to test the possibility that the inhibition of acid phosphatases is a metabolic function of molybdenum in higher plants.

II. METHODS

(*a*) *Experimental Material*

The test plant was tomato (*Lycopersicon esculentum* Mill.); Pan America and Bonny Best varieties were used and no differences due to variety were ob-

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served. Plants were grown in water culture in a glass-house. The water culture medium was of the composition recommended by Cheng Tsui (1948), except that KH_2PO_4 was substituted for K_2HPO_4 and the final pH of the nutrient solution was adjusted to 6.0 with KOH at the beginning of the experiment. In order to remove traces of molybdenum, molar stock solutions of the four salts supplying the macronutrients were extracted with 1 per cent. 8-hydroxyquinoline in chloroform at pH 3-4, as suggested by Gentry and Sherrington (1950). Salts supplying the micronutrients were recrystallized at least three times before use. Plants grew well in the above nutrient solution to which 0.05 p.p.m. of molybdenum (as Na_2MoO_4) had been added. Unless otherwise stated, material used was from molybdenum-treated, normal plants and not from molybdenum-deficient plants.

(*b*) *Preparation of Enzyme Extract*

Two types of enzyme preparations were used. One was a plant powder made from leaves according to the method of Ignatieff and Wasteneys (1936). The method involves grinding the leaves, drying the grindate at 25°C for 1 hr, and re-grinding the product to a fine homogeneous powder. The second enzyme preparation used was a cell-free brei made by grinding or blending the plant material in approximately three times its weight of N/10 succinic acid-sodium succinate buffer, pH 6.0, in the cold, filtering through muslin, and centrifuging for 4 min at 3,000g. The supernatant was dialysed overnight at 4°C against N/20 succinate, pH 6.0, and stored at -20°C. Before use, the brei was re-centrifuged at low speed to remove the clumped particles formed during storage. No alkaline phosphatase activity was detected in these preparations.

(*c*) *Measurement of Phosphatase Activity*

The activity of phosphatase preparations was determined either by measurement of the amount of inorganic phosphate liberated from the substrate, or, when PNPP was the substrate, by measuring the *p*-nitrophenol (PNP) liberated as described by Axelrod (1947). Inorganic phosphate was determined by the method of Waygood (1948), which uses ascorbic acid at pH 4.0 to reduce the phosphomolybdate. Incubated substrate controls were run with each experiment to account for non-enzymic substrate hydrolysis under experimental conditions. With β-glycerophosphate as the substrate, maximum phosphatase activity was obtained at pH 5.9-6.2, and all incubations were carried out in this pH range. The activity of the enzyme preparation was not affected by 10^{-2}M KCN, 10^{-2}M ICH_2COOK, 2.5×10^{-4}M 2,4-dinitrophenol, or 10^{-4}M *p*-chloromercuribenzoate. Sodium fluoride inhibited the enzyme preparation by 59 per cent. at 10^{-2}M.

III. RESULTS

(*a*) *Substrate Specificity and Inhibition by Molybdate*

A range of organic phosphate compounds of physiological interest was tested as potential substrates for tomato-leaf acid phosphatases in the presence and absence of added molybdate (Table 1). The range of substrates hydrolysed was essentially in agreement with the results of Axelrod (1947) for citrus fruit phosphatase, Nakamura and Becker (1951) for ragweed pollen phosphatase, and Hoagland (1952) for tomato acid phosphatase. Glucose 1-phosphate was comparatively slowly hydrolysed. Triose phosphate, which has not been tested previously, was readily hydrolysed. With all substrates tested phosphatase activity was markedly inhibited by 5 × 10^{-4}M Na_2MoO_4.

TABLE 1

ACTIVITY OF TOMATO ACID PHOSPHATASES TOWARDS A RANGE OF SUBSTRATES, AND THE EFFECT OF MOLYBDATE ON ENZYME ACTIVITY

Phosphatase preparation = dialysed leaf brei (see Methods). Substrate concentration 2×10^{-3}M. Sodium molybdate 5×10^{-4}M. Phosphatase activity = μg inorganic P liberated in 20 min at 30°C

Substrate	Phosphatase Activity	
	Without Molybdate	With Molybdate
Glucose 1-phosphate	8	Nil
β-Glycerophosphate	28	Nil
Fructose 6-phosphate	22	Nil
Fructose 1, 6-diphosphate	26	Nil
3-Phosphoglycerate	35	Nil
p-Nitrophenyl phosphate	49	7
Adenosine triphosphate	78	25
Adenosine 3-phosphate	34	3
Inosine 3-phosphate	30	5
Triose phosphate*	236	44

*Figures for triose phosphate are not strictly comparable since they were obtained from another experiment using plant powder as enzyme source, and incubated 60 min at 31°C. The rate of hydrolysis of triose phosphate was approximately equal to that of fructose 6-phosphate. Triose phosphate was prepared according to Myerhof (1938).

(*b*) *Phosphatase Activity in Relation to Molybdate Concentration*

Using β-glycerophosphate (0.02M), the activity of a phosphatase preparation was measured over a range of molybdate concentrations (Fig. 1). At this substrate concentration 6.25 × 10^{-6}M Na_2MoO_4 caused 50 per cent. inhibition.

(*c*) *Type of Inhibition Caused by Molybdate*

The construction of curves relating initial velocity of a phosphatase-catalysed reaction to substrate concentration (sodium β-glycerophosphate) in the presence and absence of inhibitor (5×10^{-6}M Na_2MoO_4) showed that molybdate inhibits the enzyme competitively, i.e. when reciprocals of both initial velocity and substrate concentration are plotted, according to the treatment of Lineweaver and Burk (1934), two straight lines are obtained having a common intercept on the *y* axis, but significantly differing slopes (Fig. 2). This result is in agreement with those of Rothstein and Meier (1949) for the surface phosphatases of intact yeast cells. From the data of Figure 2 the Michaelis constant for the enzyme-substrate complex was calculated, as described by Wilson (1950), to be 1.58×10^{-2}M, and the dissociation constant for the enzyme-inhibitor complex to be 10^{-5}M.

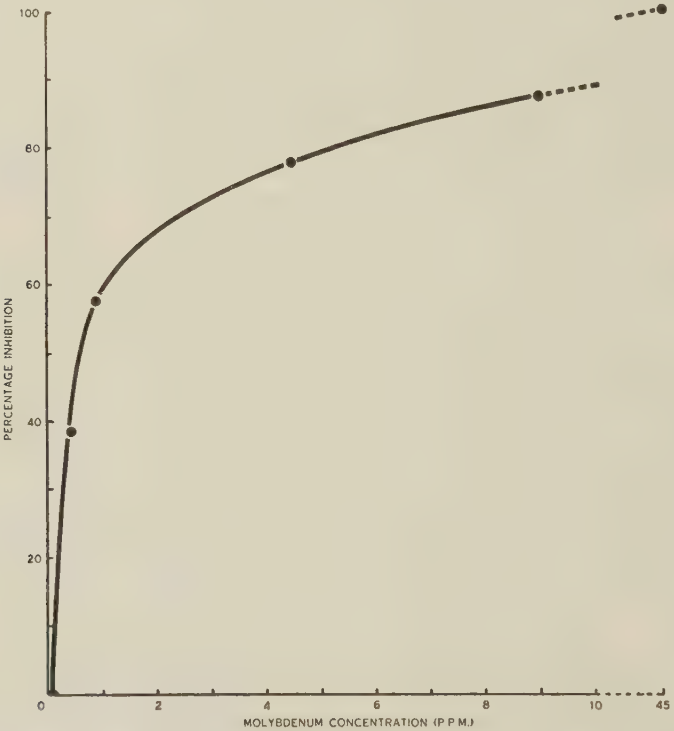

Fig. 1.—Effect of sodium molybdate on the activity of tomato acid phosphatase *in vitro*. Each tube contained 1.0 ml 0.2M glycerophosphate; 0.05 g plant powder (see Methods); the appropriate amount of Na_2MoO_4; and distilled water to make 10.0 ml. Incubated at 30°C for 30 min.

(*d*) *Measurement of the Phosphatase Activity of Intact Plant Cells*

In order to test whether the molybdate status of the plant could affect phosphatase activity *in vivo* it was first necessary to devise a technique by which the phosphatase activity of whole, intact plant cells could be measured. This was achieved by use of PNPP as substrate. If roots of intact plants are immersed in a solution of PNPP the latter is taken up and hydrolysed, and the product of hydrolysis (PNP) diffuses out into the surrounding solution. By taking aliquots of this external solution, making alkaline, and reading the optical transmittance at 400 mμ, the rate of breakdown of the substrate can be followed, and a measure is thus obtained of the activity of the phosphatases of the intact root cells.

The following experiment illustrates this fact: molybdenum-deficient plants 5 wk old were removed from water cultures, roots were washed first in running tap water, and then in distilled water, and each plant transferred to a beaker containing 20 ml of 5×10^{-3}M PNPP, the roots being immersed in the solution. The solution of substrate was adjusted to pH 6.0, and, although unbuffered, the pH did not change more than 0.2 units during the experiment. After 2 hr the roots were washed, and the external solution and the roots were analysed for PNPP and PNP. Roots were ground in 2.5 per cent. trichloracetic acid and residual PNPP estimated as the increase in PNP after hydrolysis in 3N HCl for 3 hr on a boiling water-bath (Bessey and Love 1952). Roots of a plant similarly treated and stood in water for the same period were used to correct for transmittance due to endogenous absorbing substances. Analyses showed (Table 2) that 96 per cent. of the PNP could be recovered in the external solution, so that sampling the external solution for PNP provides a reliable means of determining the rate of the reaction.

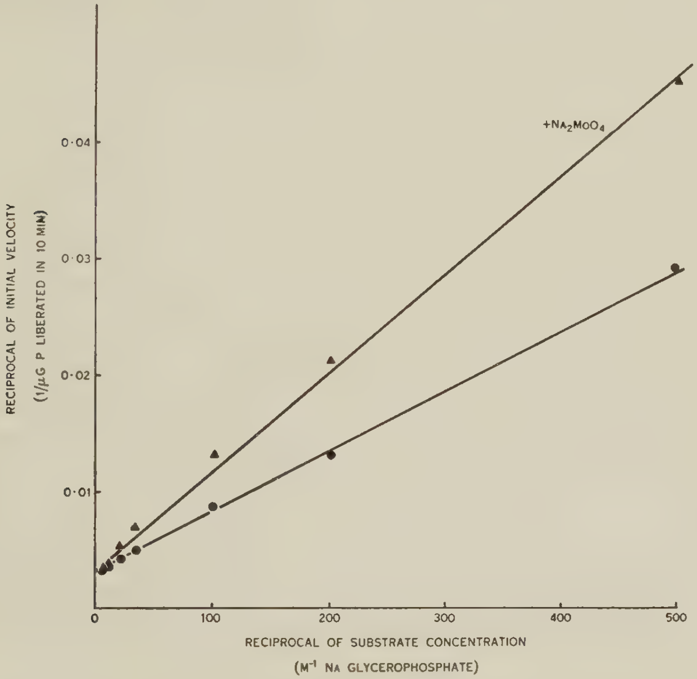

Fig. 2.—Competitive inhibition of tomato acid phosphatases by sodium molybdate. The common origin of the two lines on the *y* axis is significant at $P = 0.07$. Each tube contained 0.05 g plant powder (see Methods); appropriate concentrations of β-glycerophosphate; 5×10^{-6}M Na_2MoO_4 in the curve indicated; and distilled water to make a total volume of 5.0 ml. Incubated 10 min at 30°C.

Microscopic examination and plating of root segments on potato dextrose agar failed to disclose any contamination by microorganisms. The experiment was carried out in the glass-house and a solution of PNPP was held under the same conditions and analysed as above in order to account for non-enzymic hydrolysis. When solutions were shielded from light, non-enzymic hydrolysis was slight.

TABLE 2

UPTAKE AND HYDROLYSIS OF PNPP BY WHOLE TOMATO ROOTS IN THE PRESENCE AND ABSENCE OF MOLYBDATE

External Medium (Total vol. 20 ml)	PNPP in Roots (μM/g fresh wt.)	PNP in Roots (μM/g fresh wt.)	PNP in External Solution (μM/g fresh wt.)	$\frac{\text{PNP in External Solution}}{\text{Total PNP Formed}} \times 100$
PNPP (5×10^{-3}M)	0·86	0·68	16·3	96
PNPP (5×10^{-3}M)+ Na_2MoO_4 ($7{\cdot}5\times10^{-4}$M)	2·02	0·19	4·61	96

(*e*) *Effect of Molybdate on the Activity of Phosphatases* in vivo

Using the technique described in the preceding section, the effect of molybdate on the phosphatases of intact roots of 5-wk-old molybdenum-deficient plants was tested. Figure 3 shows the inhibitory effect of molybdate added to the external solution on the liberation of PNP into this solution. The volume of each aliquot (0.2 ml) taken from the external solution was small in comparison to the total volume (20 ml), and hence the concentrating effect of withdrawing aliquots can be ignored. Analysis of the roots for PNP and unhydrolysed PNPP showed that in the presence of molybdate, roots contained more PNPP than in its absence; i.e. the hydrolysis of PNPP to PNP had been markedly inhibited (Table 2). Thus *in vivo* as well as *in vitro*, molybdate inhibits tomato phosphatases.

(*f*) *Phosphatase Activity of Roots in Relation to the Molybdate Concentration of the External Solution*

This was measured by the rate of appearance of PNP in the external solution when roots of whole plants were immersed in a PNPP solution (10^{-4}M) containing varying concentrations of molybdate. At the end of an experiment roots were removed from the plant, washed, and dried at 100°C, and the phosphatase activity was expressed as the PNP concentration of the external solution per unit dry weight of roots. Plants used were 4-5 wk old, and molybdenum-deficient. Results (Fig. 4) showed that the phosphatases of intact tomato roots were inhibited by 50 per cent. when the concentration of molybdate in the external solution was approximately 6×10^{-6}M. Significant inhibition was obtained with 10^{-7}M Na_2MoO_4 ($\equiv$0.0096 p.p.m. Mo).

In order to obtain a measure of the molybdate concentration at the site of phosphatase activity in the root cells, a cell-free brei was made from the roots of plants from the same batch as those used in the above experiment. The phosphatase activity of this preparation was measured under the same conditions of substrate and molybdate concentrations as used in the above "whole root" experiment, and the degree of inhibition by molybdate was compared. It will be seen (Fig. 4) that the order of inhibition of the cell-free preparation was the same as that for the intact root system.

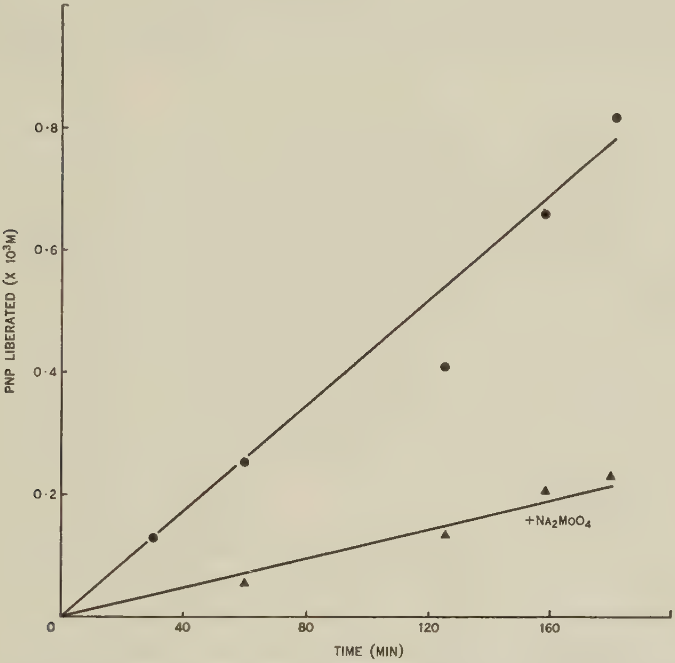

Fig. 3.—Effect of molybdate on the hydrolysis of PNPP by the phosphatases of intact tomato roots. Substrate 5×10^{-3}M PNPP; Na_2MoO_4 7.5×10^{-4}M. Total volume 20 ml.

IV. DISCUSSION

Experiments reported here were designed to examine the possibility that molybdate normally functions as a metabolic inhibitor of acid phosphatases in plants. Results showed that acid phosphatase preparations from tomato were significantly inhibited by concentrations of molybdate as low as 10^{-7}M (Figs. 1 and 4). This is within the physiological range of molybdenum concentrations normally found in higher plants (Williams and Moore 1952), and together with the possibility of local concentrations of molybdenum within the cell (Whatley, Ordin, and Arnon 1951), indicates that molybdate could significantly inhibit phosphatases in intact cells providing it were present at the sites of phosphatase action.

The inhibition is shown to be competitive (Fig. 2), and hence its degree will vary with the substrate concentration. Since neither the natural substrates of plant phosphatases nor their concentrations under physiological conditions are known, it is not possible from a knowledge of the molybdenum status of the plant to predict the extent of inhibition *in vivo*. It was therefore necessary to test the hypothesis with intact living cells. This was achieved by measuring the hydrolysis of PNPP by whole roots of tomato in the presence of graded amounts of molybdate. These experiments (Table 2; Fig. 3) showed that inhibition of phosphatase by physiological concentrations of molybdate does occur, at least in the special case of the root cells. Whether this result can be extrapolated to other plant tissues is not known. Evidence that this is a possibility is given by the data of Figure 4, which shows that inhibition by molybdate was

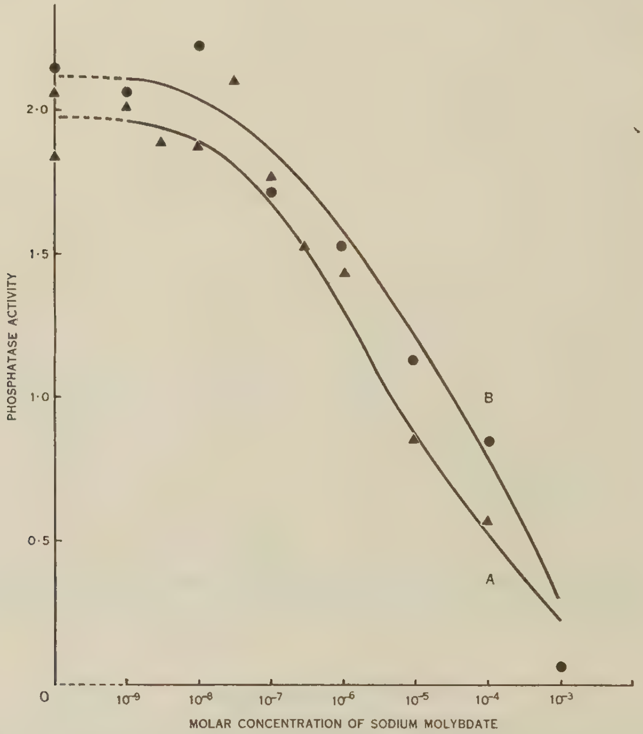

Fig. 4.—Effect of molybdate concentration on the *in vivo* and *in vitro* phosphatase activity of tomato roots. *A* (▲) = intact roots; activity expressed as PNP liberated per 0.1 g dry wt. of root in 60 min. *B* (●) = cell-free root brei; activity expressed as concentration PNP liberated in 20 min at 30°C.

of the same order in both "whole root" and "root brei" experiments. Since significant inhibition was obtained with concentrations of 10^{-7}M in both cases, and since this is of the order of physiological concentrations of molybdenum, it does suggest that a similar inhibition of phosphatases could occur in other plant tissues.

Attempts to introduce PNPP into the leaves by immersing cut stems in a solution of the substrate, a technique used by Rautenen (1948) to follow assimilation of amino acids, were unsuccessful. The very high blank due to endogenous substances which absorb strongly at 400 mμ made it impossible to measure the relatively small change due to the formation of PNP under these conditions.

Bossard (1947) has shown that the effect of molybdate on phosphatases is specific, and that molybdate does not affect the activity of β-glucosidases, proteases, lipase, oxidases, peroxidase, catalase, dehydrogenases, and carboxylases found in extracts of plant tissues. In addition, potato apyrase is unaffected by sodium molybdate concentrations as high as 5×10^{-2}M (Rosenberg, personal communication).

If the hypothesis is correct it should follow that the phosphatase activity of molybdenum-deficient plants is higher than that of plants with adequate molybdenum. However, a comparison of the phosphatase activity of intact roots of normal and deficient plants on a protein basis would not provide an unequivocal answer to the question of the possible *in vivo* effect of molybdate on phosphatase activity, since it is known that the relative content of any one enzyme in a plant tissue may vary with its physiological stage and nutritional state. Axelrod and Jagendorf (1951) demonstrated that phosphatase, catalase, and invertase activities of detached leaves (per unit dry weight) remained at their original level for 14 days, in which time the protein content of the leaves decreased by 45 per cent. As has been emphasized by Wood (1953), in such a case as the present one it is only valid to compare the enzyme activities of physiologically uniform material.

Indirect evidence of increased phosphatase activity, relative to other enzymes, in molybdenum-deficient plants is given by the fact that in the author's experience the inorganic phosphate content of the leaves per unit dry weight is from four to ten times greater than that of leaves from normal plants, both series being grown in water culture with identical phosphorus nutrition. On a protein basis this ratio would be further widened since the molybdenum-deficient plants were found to contain low concentrations of protein. Furthermore, it has been found (Possingham, personal communication) that upon addition of molybdate to deficient plants there is a rapid redistribution of phosphate from the inorganic to the organic fractions. Also Wilson and Cutter (1952), in a histochemical study of developing coconut, demonstrated the coincidence of high inorganic phosphate concentrations with sites of increased phosphatase activity. Although indirect, this evidence does support the hypothesis of increased phosphatase activity in molybdenum-deficient plants.

V. ACKNOWLEDGMENT

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A GRANULOSIS VIRUS ATTACKING THE LARVAE OF *PERSECTANIA EWINGII* WESTW. (LEPIDOPTERA : AGROTIDAE) IN SOUTH AUSTRALIA

By H. F. Lower*

[*Manuscript received January* 8, 1954]

Summary

An epizootic of a virus disease in a larval population of *Persectania ewingii* Westw. (Lepidoptera : Agrotidae) in South Australia is described, and possible methods of its transmission in the population are suggested. The symptomatology of the disease during its progress is outlined. A description of the virus and its structure follows. The aetiology of the disease is described, and the paper concludes with a brief history of 5000 diseased larvae which were collected in the field.

I. Introduction

In the course of investigations into the biology of *Persectania ewingii*, the author came upon a severe epizootic of virus disease in a dense population of the larvae inhabiting a little valley near the township of Cherry Gardens, in the Mt. Lofty Ranges, some 20 miles south of Adelaide. The population was an isolated one, no other larvae of this species being found within 20 miles of the locality.

The area is typical tussock grassland (*Stipa pubescens* R.Br. the dominant), with a few scattered gum trees, and the larvae had eaten practically all the current season's growth of grass. There remained chiefly the tall, dry stalks which, in the previous year, had borne inflorescences, and each stalk carried one or more dead larvae.

II. Method of Sampling, and Composition of Population

Numerous living larvae, principally in their two final instars (fifth and sixth) were found in masses beneath frass and debris surrounding the bases of the grass plants, or under bark on the ground. To determine the degree of infection, 5168 larvae were collected, some from every type of minor habitat within the area.

These comprised 5141 recognizably diseased fifth and sixth instar larvae, and 27 fourth instar larvae which, although showing no symptoms of the disease at the time, developed it later. This uniformity of age composition of the population is characteristic of first generation larvae of this species. In "outbreak years," the adults emerge from their pupae in May, and the females oviposit and die within a fortnight. This results in the majority of larvae, in any one area, being within an instar of each other during development. The small number of younger larvae probably resulted from delayed emergence of a few

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adults. The larvae were brought back to the laboratory where 168 moribund specimens were used as a source of virus for infecting healthy larvae. The remaining 5000 were placed in cages for observation.

III. Transmission of the Virus

It was found that if larvae in any instar, from known healthy laboratory colonies, were placed in a cage with diseased larvae, or in one from which diseased larvae had recently been removed, all contracted the disease. Under these conditions, the possible modes of transmission appeared to be the eating of food contaminated with (1) body fluid from dead larvae, (2) saliva left on food by diseased larvae, (3) faeces of diseased larvae. In the field there are two additional possibilities: (4) the cannibalistic habits of starving larvae, and (5) through the agency of the ovipositors of parasitic Hymenoptera. These possibilities, except the fourth, were separately investigated. Transmission through cannibalism could not be tested since the disease is so infectious that proven agents of transmission could not, at the same time, be excluded. From the tests carried out, however, it appears certain that infection would follow the feeding of a healthy larva on a diseased one.

Attempts to inoculate healthy larvae, by piercing them with a needle previously thrust into diseased ones, met with varying success. The fact that results were sometimes positive, however, indicates that the ovipositor of hymenopterous parasites may be a significant agent in transmitting the virus from one population to another.

Method 1

Diseased larvae, after being washed several times with distilled water, were ground in a mortar with a little distilled water. The mixture was filtered to get rid of gross particles. The slightly opalescent, yellowish brown filtrate was sprayed on fresh grass in a sterilized cage, and 50 healthy larvae in various instars were allowed to feed on it for 24 hr. They were then divided into five equal batches, placed in clean cages, and given the same treatment as those in the laboratory colonies from which they came. Every larva was infected, and all except two died in the fifth instar. The two which survived were in the fifth instar when infected. These ultimately produced moths.

Method 2

Grass on which diseased larvae had fed was washed with a little distilled water, the liquid filtered, and the filtrate sprayed on clean grass. The procedure of the first method was then followed. Twenty-four larvae were infected (3 — 6 — 1 — 7 — 7) and later died; the remaining 26 showed no disease symptoms, and subsequently pupated. One pupa failed to develop; no cause for this was found. Twenty-five apparently normal moths emerged from the remainder. It is necessary to point out here that, as larvae which are recognizably diseased do not feed, larvae which had been infected by the first method were used. As these all died later from the virus, it was assumed that they were infected at the time that the test was applied.

Method 3

Frass, from recognizably diseased larvae, was ground with distilled water, filtered, and the filtrate used as in the first method. The results were inconsistent; in the five batches the infected specimens were 0 — 0 — 0 — 7 — 1.

The above results suggest that, in nature, infection through the ingestion of food contaminated with either virus-containing saliva, or frass, is important in the transmission of virus among the individuals of a population. While the entry of body fluid, by any means, into the body of a larva, appears to cause certain infection, it is not normally present until some larvae have died. By this time, the part of the population which will contract the disease appears to be already infected.

IV. Symptomatology of the Disease

Young diseased larvae, and all healthy ones, have a bold colour pattern, consisting of a series of narrow, longitudinal stripes of yellow, white, and orange, on a velvety, brownish black background. No symptoms are observable until infected larvae reach their fifth or final instars. When symptoms appear in fifth instar larvae, these invariably die. When symptoms are delayed until the sixth instar, most larvae die but a few complete their development. Time of infection appears to be decisive here.

(*a*) *First Stage*

The initiation of the virulent phase of the disease is marked by cessation of feeding, and restlessness. The larva climbs a grass stalk or similar object, and then descends. It may do this many times until a suitable place is found. It then takes up an inverted position which it maintains by hooking the crochets of the terminal larvapodia in small crevices in the stalk. The final site is apparently determined by the object's having the necessary roughness to enable the crochets to take a firm hold. Pieces of narrow glass rod, straws thinly coated with hard paraffin, or thin sticks smoothed with emery cloth and painted with shellac are repeatedly climbed but never selected. A favoured position is where a leaf or a pedicel grows out from the stem. In cages, the metal gauze at the ends is most frequently chosen. Once in position, the larva contracts so that it is shorter and stouter than a healthy resting larva of the same size. Soon the whole of the body weight is supported by the terminal larvapodia, and in consequence the posterior segments become fully extended (Fig. 1*A*). The larva shown in Plate 1, Figure 1, is typical and was the model from which the series of text figures was copied. If disturbed, the larva will crawl down the stem and ascend another, but normally it remains where it has attached itself.

(*b*) *Second Stage*

Other changes now follow in quick succession; within 12 hr, the colour pattern has faded. The cuticle assumes a dull lead-grey coloration and becomes wax-like and translucent. Liquefaction of the body contents begins at the anal end so that the insect becomes suspended by the cuticle of the terminal segments.

The anterior part of the body becomes distinctly swollen (Fig. 1*B*). Even in this stage, locomotion is still possible. If sufficiently stimulated, the insect will slowly crawl down to the soil surface, but is unable to re-ascend. It merely climbs any object sufficiently high for its head to be above the soil when suspended, or dies on the soil surface. In the field, the majority of the larvae were in this condition, probably having been dislodged by high winds which had blown a day or so before the area was examined.

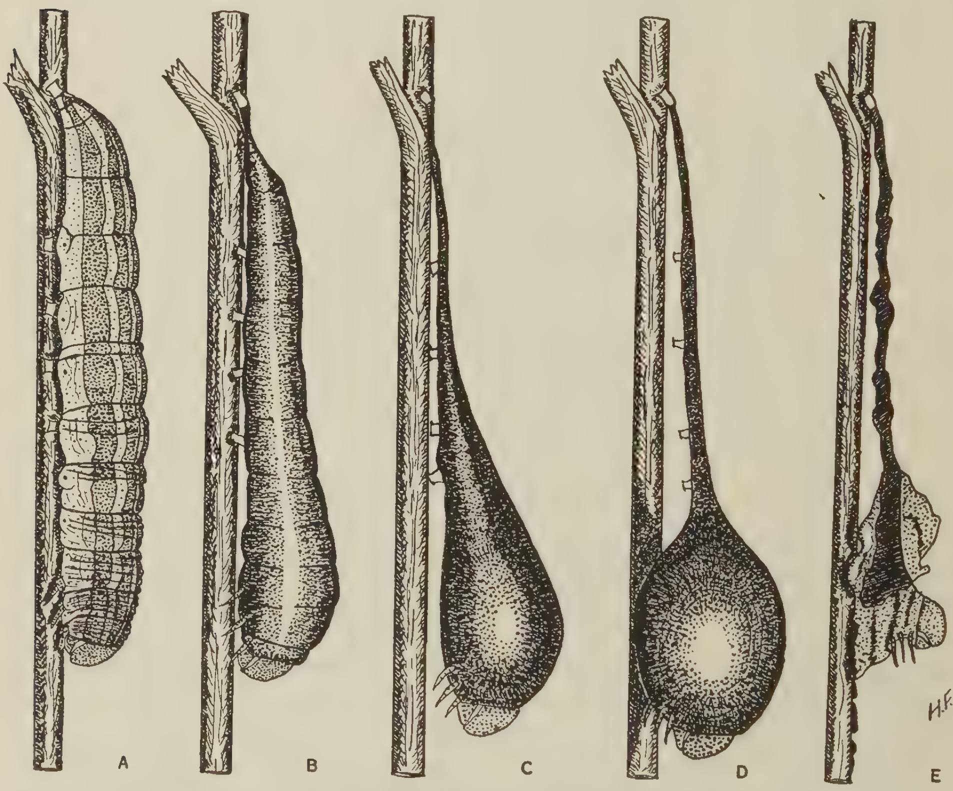
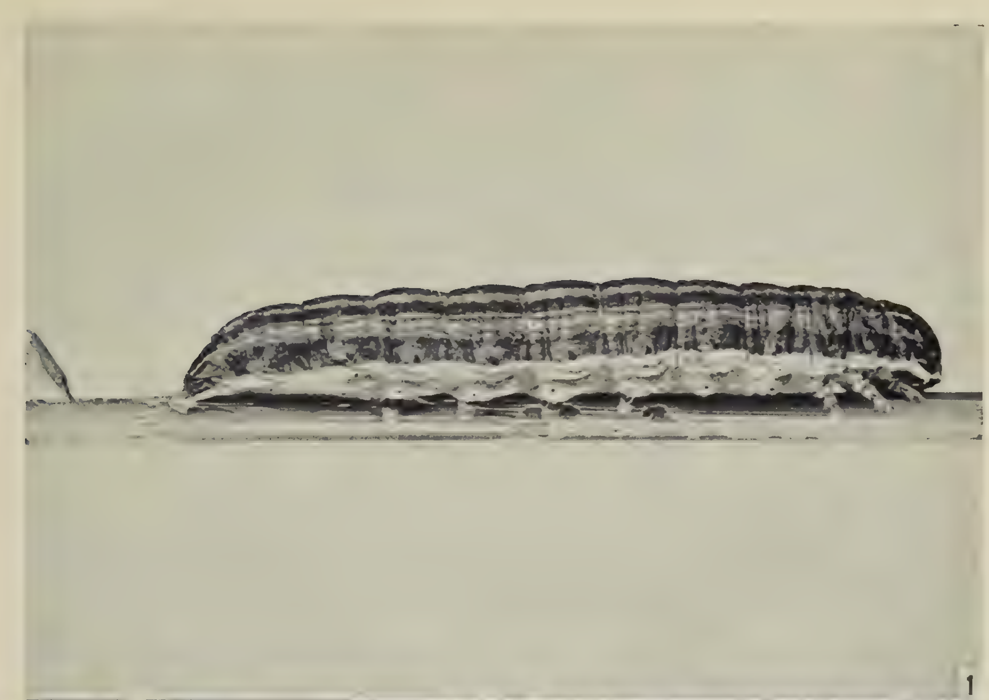


Fig. 1.—*A*, larva in position of final attachment. First stage of virulent phase. *B*. larva in second stage of virulent phase. Note colour changes and beginning of liquefaction at anal end. *C* and *D*, larva in third stage of virulent phase. In *D*, liquefaction is complete, *E*, rupture of cuticular sac and escape of dark brown body fluid.

(*c*) *Third Stage*

Death occurs within 24 hr, with a progressive onset from posterior to anterior. Specimens are not uncommon which can still move the head and thoracic legs, but are incapable of moving any part of the body posterior to these. Liquefaction proceeds rapidly. The cuticle becomes uniformly blackish brown, and the anterior of the insect is distended to form a fluid-containing sac (Fig. 1*C*). Within a further 24 hr, liquefaction is complete, the swollen anterior being supported by the shrunken, collapsed cuticle which becomes



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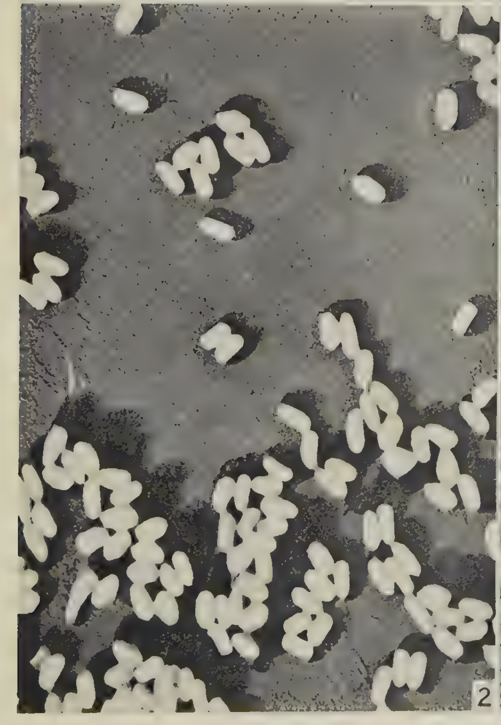

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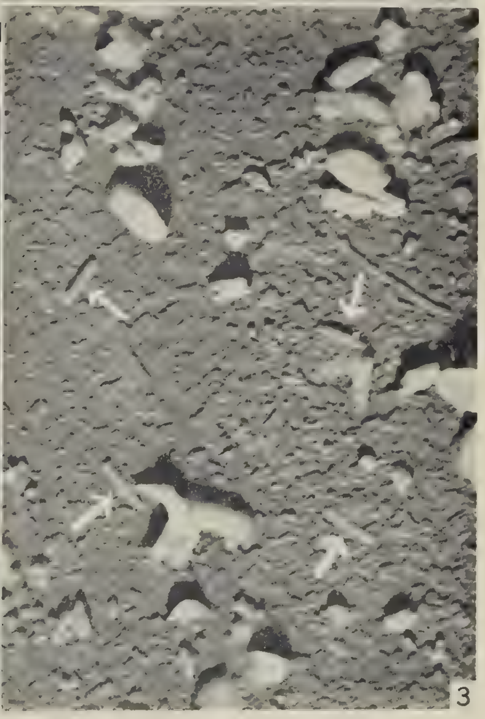

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delicate, and easily fractures (Fig. 1*D*). Soon, the sac ruptures, permitting the escape of a dark brown, odourless fluid. The dried, blackened cuticle remains attached until blown away by the wind or washed down by rain (Fig. 1*E*).

V. Internal Histological Changes and Description of the Virus

On dissection of a larva in the first stage of the virulent phase, the fat-body is seen to be greatly enlarged. The fatty material found in the fat-body of a healthy larva has largely disappeared and a dirty, whitish, opaque suspension fills the distended cells. Microscopic examination, under oil immersion, shows that the original contents of the cells have degenerated. The liquid which replaces them consists of numerous minute granules showing Brownian movement, suspended in an apparently colourless, clear fluid. No structural details of the particles can be observed.

(*a*) *The Granules*

Electron micrographs show the granules to be subovoid, elongate bodies with more or less parallel sides. They often appear to be united end to end in chains, but this is possibly an artifact (Plate 1, Fig. 2).

If treated with alkali, the granules swell and then rupture, and each is found to consist of a proteinaceous envelope enclosing a single rod of virus (Plate 1, Fig. 3).

This encapsulated virus body is typical of the granulosis group of insect viruses for which Steinhaus (1949) erected the genus *Bergoldia.* From the morphology of the granules, the species would appear to be closely allied to an as yet unnamed one found by Hughes and Thompson (1951) in the omnivorous looper (*Sabulodes caberata* Gn.).

The type material of the virus is retained in the collection of the University of California, Laboratory of Insect Pathology, Albany, California, U.S.A., where it will be available for eventual taxonomic study.

VI. Aetiology of the Disease

At present, there is no evidence to show how initial infection of the population occurred. Once this had taken place, however, certain predisposing factors may account for its rapid spread and for the high incidence of mortality in the area.

(1) The density of larval population was very high. At night no larva could wander more than a few inches without coming in contact with a neighbour, or crossing many times over the tracks of diseased larvae. The larval habit of congregating by day in dense masses round the bases of grass plants, down the stems of which body fluid from dead larvae was constantly being washed by rain and dew, placed them in a position ideal for being infected. Such contaminated grass, being the only available food, tended further to attract the larvae. The importance of crowding may be gauged from the fact that though

this species is subject to attack over wide areas, only an occasional larva contracts the disease when the population density is low.

(2) All larvae showed evidence of starvation; specimens which fed voraciously when supplied with food in the laboratory had very little food in the gut when collected. This condition would probably tend to lower their resistance to infection.

(3) Rainfall in the area is high (30 + in. average), and falls principally during the period of development of the species. While the epizootic was in progress both rainfall and number of cloudy days were much above average so that the larvae were exposed almost constantly to a mild, very humid atmosphere. In another locality where population density was at least as high, but where the rainfall for the corresponding period was only 11 + in., and bright sunny days were of frequent occurrence, not a single infected larva was found. There is, of course, the possibility that, in this case, the virus had not been introduced into the population.

VII. History of the Collected Larvae

Of the 5000 larvae collected, 27 mid-instar larvae showed no symptoms of the disease at the time of collection, but all developed it later; 4803 larvae died, and 195 attempted to pupate on the soil surface below litter. No cell-building in the soil, a characteristic of the normal larva when preparing to pupate, occurred. Two larvae could not be accounted for. The 195 which attempted pupation produced 51 adult moths and 144 parasites. The moths laid several thousand eggs but the larvae which hatched from these died in their third and fourth instars from a disease produced by a species of the *Bacillus cereus* group (identified by Dr. C. G. Thompson) before the presence or otherwise of virus could be determined.

Two interesting facts emerge from these results. The first is that the virus is not necessarily fatal to every infected larva. Dr. Thompson informs me that he has found that if a virus disease is contracted by the larvae of some species towards the end of their larval period, some may complete their development. This applies also to *P. ewingii*.

The second significant fact is that some parasites at least are immune to the virus. Of the 144 adult parasites, 11 were Hymenoptera (Braconidae) and 133 were Diptera (four species of Tachinidae). While the marked preponderance of Diptera may indicate that the larvae of *P. ewingii* are not preferred hosts of the Hymenoptera, there is also the fact that this order tends to parasitize first and second instar larvae, whereas the Tachinidae prefer later instars. Hymenopterous parasites would thus be exposed for a longer period to conditions which might not favour their development than would the dipterous. Much more information is required, however, before any definite verdict on these matters can be given.

VIII. Acknowledgments

I desire to acknowledge my indebtedness to Dr. Clarence G. Thompson, Insect Pathologist, University of California, for all the information concerning the structure and classification of the virus. He has kindly authorized my use of this information and of the electron micrographs, which are the work of Mr. K. M. Hughes of the same institution, to whom also I express my thanks.

IX. References

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Explanation of Plate 1

Fig. 1.—Final instar larva of *Persectania ewingii* in position of attachment (on vertical grass stem). The terminal larvapodia are hooked on the stem, the posterior segments are extended, and the anterior segments contracted.

Fig. 2.—Granules. × 14,600. (Photo by courtesy of C. G. Thompson.)

Fig. 3.—Granules after treatment with alkali. The granules have ruptured. Arrows indicate the liberated virus bodies. × 20,800. (Photo by courtesy of C. G. Thompson.)

STUDIES ON CHITIN

I. ENZYMIC DEGRADATION OF CHITIN AND CHITIN ESTERS

By R. H. Hackman*

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Summary

A study has been made of the enzymic degradation of chitin and the sodium salt of chitin sulphuric acid by a chitinase prepared from the intestinal tract of the snail, *Helix aspersa*. It is shown that, in a citrate-phosphate buffer, the pH for optimum activity is 4.8 for both substrates. Chitin, prepared from both lobster cuticle and fly puparia, is broken down by snail chitinase to *N*-acetyl-D-glucosamine, although there is also a trace of D-glucosamine. The sodium salt of chitin sulphuric acid also breaks down to *N*-acetyl-D-glucosamine and D-glucosamine. In both cases *N*-acetyl-D-glucosamine was isolated. The chitinase is without action on chitin nitrate. Acid hydrolysis of both chitin nitrate and the sodium salt of chitin sulphuric acid leads to the formation of D-glucosamine, which has been isolated as 2-hydroxynaphthylidene glucosamine. All attempts to phosphorylate chitin were unsuccessful. The structure of chitin is discussed.

I. INTRODUCTION

The work described in this paper is part of an investigation into the metabolic sources of chitin. In arthropods there must be very active systems both for the synthesis and for the solubilization of chitin because the cuticle is largely solubilized, reabsorbed, and then formed anew at each moult. No precursors of chitin are known although there are suggestions in the literature that the chitin arises from the transformation of glycogen, protein, and cellulose (for references see Richards 1951). However, the information is not sufficiently definite to warrant speculation on possible metabolic sources.

Chitin is a component of arthropod cuticles, it occurs in a number of other groups of animals and in certain fungi (Richards 1951). It is a high-molecular-weight polymer of *N*-acetyl-D-glucosamine (more correctly 2-acetamido-2-deoxy-α-D-glucopyranose) residues, linked in the 1,4-β-glucosidic manner of cellulose. Chitin, as usually prepared for chemical studies, is a colourless amorphous solid, insoluble in water, dilute acids, dilute and concentrated alkalis, alcohol, and all organic solvents. It is soluble in concentrated mineral acids, generally with some degradation to lower saccharides. When chitin is treated with alkali hydroxides at high temperatures (150-160°C) the acetyl groups are detached and the polysaccharide chitosan is formed. In "purified" chitin there is usually a strong attraction between adjacent molecular chains, as shown by its resistance to dispersion, but the nature of the bond involved is not known.

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It has been known for some time that many bacteria produce enzymes which will digest chitin, and such bacteria have been isolated from fresh water, sea-water, salt beds, mud, soil, decomposing manure, and from the gut contents of many marine, freshwater, and terrestrial animals (for review see Richards 1951). A recent paper by Veldkamp (1952) describes the isolation of two strains of bacteria from manured garden soil which are capable of decomposing chitin to *N*-acetyl-D-glucosamine, D-glucosamine, and ammonia. The enzymic breakdown of chitin has been studied in some detail by Karrer and his colleagues (Karrer 1930; Karrer and Hoffmann 1929; Karrer and François 1929), using a chitin-splitting enzyme obtained from digestive juices of vineyard snails (*Helix pomatia*). The hydrolysis of chitin by this chitinase was found to proceed best at pH 5.2 and from fungal chitin as much as 80 per cent. of *N*-acetyl-D-glucosamine together with no more than a trace of D-glucosamine was obtained. Yonge (1932) has shown that the digestive juices from the snail *Helix aspersa* are capable of dispersing lobster cuticle. In recent publications Jeuniaux (1950*a*, 1950*b*, 1950*c*) has described the isolation of bacteria from the gut of a snail (*H. pomatia*) which is capable of solubilizing chitin. These bacteria produce an extracellular chitinase, no chitinolytic enzymes being found in the snail hepatopancreas.

Sulphuric acid esters of chitin have been prepared and their use as synthetic anticoagulants studied by a number of workers (Bergström 1935, 1936; Karrer, Koenig, and Usteri 1943; Astrup, Galsmar, and Volkert 1944; Piper 1946). Several different nitrate derivatives of chitin have been made and their structures investigated (von Fürth and Scholl 1907; Schorigin and Hait 1934; Clark and Smith 1936; Meyer and Wehrli 1937).

II. Materials and Methods

(*a*) *Preparation of Chitin*

Lobster shell was cleaned by washing and scraping under running water and dried in an oven at 100°C. Clean and dried lobster shell (220 g) was digested with 2N aqueous hydrochloric acid (2 l.) for 5 hr at room temperature, washed well, dried at 100°C (wt. 91.3 g), and ground to a fine powder in a hammer mill. The finely ground material was extracted for 48 hr with cold 2N aqueous hydrochloric acid (500 ml), the contents of the flask being vigorously agitated at frequent intervals. The material was collected by centrifugation, washed with water, and extracted with 1N aqueous sodium hydroxide solution (500 ml) for 12 hr at 100°C, the contents of the flask again being shaken at frequent intervals. The insoluble material was collected by centrifugation and again extracted with aqueous alkali for 12 hr at 100°C. The material was further extracted with alkali three times. The insoluble material was collected by centrifugation, washed with water (by centrifugation) until of neutral reaction, then with ethanol and ether, and dried *in vacuo* over phosphorus pentoxide. Yield: 37.4 g (17 per cent.) of a cream-coloured powder (Found: N, 6.8% and ash nil. Calc. for $(C_8H_{13}O_5N)_x$: N, 6.9%).

In a similar manner chitin was prepared from the puparia of *Musca domestica* L. This sample of chitin, which was a pale brown powder, had N 6.5 per cent. and ash 5.8 per cent.

(*b*) *Preparation of the Sodium Salt of Chitin Sulphuric Acid*

Chlorosulphonic acid (9.0 ml) was added with cooling to pure dry pyridine (60 ml). To this mixture was added finely powdered chitin (lobster) (6 g) and the solution heated on a steam bath for 1 hr. The solution was cooled, ice water (200 ml) and 2N aqueous sodium hydroxide solution (120 ml) added, and the sodium salt of chitin sulphuric acid precipitated by the addition of ethanol (600 ml). The suspension was left in the refrigerator overnight, the precipitate collected by filtration, dissolved in water (200 ml), and dialysed against running water for 2 days. The solution was concentrated under reduced pressure and in an inert atmosphere to 100 ml, filtered through asbestos pulp, and the sodium salt precipitated by the addition of ethanol (100 ml). The salt was collected by centrifugation, washed with ethanol and ether, and dried *in vacuo* over phosphorus pentoxide. Yield: 7.7 g of a colourless solid. Tests for chloride and sulphate ions were negative (Found: S, 11.2%).

(*c*) *Preparation of Chitin Nitrate*

Finely powdered chitin (2.0 g) was mixed to a slurry with a small volume of fuming nitric acid (sp. gr. 1.5) while cooling the mixture in ice water. More fuming nitric acid was added (50 ml total vol.) and the mixture stirred until all the chitin was in solution (10 min). The solution was kept at room temperature for 2 hr and then poured slowly into ice water (1 l.). The precipitate was collected by centrifugation, washed with water until the washing had a neutral reaction, then with alcohol and ether, and finally dried to constant weight at 110°C. Yield: 2.0 g of a colourless powder (Found: C, 33.1; H, 4.2; N, 12.1%).

(*d*) *Phosphorylation of Chitin*

Attempts to phosphorylate chitin by the method used by Karrer, Koenig, and Usteri (1943) for phosphorylating starch were unsuccessful even when the reaction was carried out at 100°C. In every case the chitin was recovered in quantitative yield and it contained no phosphorus.

(*e*) *Preparation of Snail Enzyme*

Snails (*Helix aspersa* Müller) were collected at Canberra and starved for several days so that the gut was emptied of food. The snails were covered completely with water in a closed dish so that they were killed by lack of air. By use of this method the snails came out of their shells and extended themselves completely. The shells were removed and the intestinal tracts carefully removed without loss of intestinal contents. The collected intestines were ground in a mortar with toluene and sand, washed with a little water, and filtered through asbestos pulp. The brown filtrate was dialysed against running water for 3 days and diluted to such a volume that each 2 ml represented the enzyme obtained from one snail. A few drops of toluene were added as a preservative. The preparation contained, besides a chitinase, an enzyme which attacked the cellophane membrane during dialysis. To prevent rupture of the membrane with consequent loss of material the solution was transferred to a new membrane each day.

(*f*) *Filter Paper Partition Chromatography*

One-dimensional filter paper partition chromatography (capillary ascent method of Williams and Kirby (1948)) was used for the detection of the products resulting from the action of the above chitinase preparation on chitin, the sodium salt of chitin sulphuric acid and chitin nitrate. The following two systems were used as solvents: phenol 74 per cent. and water 26 per cent. (w/w), and *n*-butanol 77 per cent., acetic acid 6 per cent., and water 17 per cent. (v/v). Whatman No. 1 filter paper was used, the chromatogram chambers were kept in a room maintained at 25 ± 1°C and the chromatograms were run overnight.

Finely powdered chitin or chitin derivative (20 mg), water (2 ml), McIlvaine (1921) citrate-phosphate buffer (pH 4.8) (2 ml), snail enzyme preparation (1 ml), and toluene (3 drops) were shaken continuously for 3 days at 25 ± 0.5°C. The mixture was centrifuged and 5 μl and multiples of 5 μl of the clear liquid were used for each chromatogram. Two controls were run for each preparation, the above mixture without enzyme and the above mixture without chitin or chitin derivative. For purposes of comparison pure D-glucosamine and *N*-acetyl-D-glucosamine were included in each chromatogram. Ninhydrin, aniline hydrogen phthalate (Partridge 1949), and the hexosamine reagent of Partridge (1948) were used as detecting agents. For *N*-acetyl-D-glucosamine better colour development was obtained if the paper was sprayed with alkali (0.1N alcoholic sodium hydroxide) before treatment with the *p*-dimethylaminobenzaldehyde reagent.

(*g*) *Enzymic Degradations*

(i) *Chitin.*—Since it was not found possible to obtain a reproducible product by the reprecipitation of chitin from cold concentrated hydrochloric acid according to the method of Karrer and Hoffman (1929), finely powdered chitin was used in all the work described in this paper. Lack of reproducibility was shown by differing rates of enzyme action.

To determine the optimum pH for the production of *N*-acetyl-D-glucosamine chitin (20 mg), water (2 ml), McIlvaine citrate-phosphate buffer (2 ml, pH range 4-7), enzyme preparation (1 ml), and toluene (3 drops) were shaken continuously for 3 days in a sealed tube at 25 ± 0.5°C. The mixture was centrifuged and 1-ml samples were taken for the estimation of *N*-acetyl-D-glucosamine and D-glucosamine. The solution from each tube was analysed in duplicate and each pH was done in duplicate. *N*-acetyl-D-glucosamine and D-glucosamine were estimated by a modification of the methods of Morgan (Elson and Morgan 1933; Morgan and Elson 1934; Aminoff, Morgan, and Watkins 1952). To develop maximum colour in the above buffered solutions it was found necessary to add 0.5 ml 0.5N aqueous sodium carbonate solution for the estimation of *N*-acetyl-D-glucosamine and 1.5 ml for the estimation of D-glucosamine. It was observed that the time and temperature of heating and the rate of cooling influenced the intensity of colour developed and although these conditions were standardized a control containing 0.1 mg *N*-acetyl-D-glucosamine was included so that a correction could be made for any variation in colour development. For *N*-acetyl-D-glucosamine the colour intensity was read at 560 mμ and for D-glucosamine at 530 mμ. Standard curves were prepared by the use of known amounts of *N*-acetyl-D-glucosamine and D-glucosamine in buffered solutions. The presence of D-glucosamine had no effect on the estimation of *N*-acetyl-D-glucosamine but *N*-acetyl-D-glucosamine interfered in the estimation of D-glucosamine. To correct for this effect a standard curve was prepared, estimating *N*-acetyl-D-glucosamine by the D-glucosamine method. *N*-acetyl-D-glucosamine and D-glucosamine were found to be stable in the presence of the chitinase preparation at least for the duration of these experiments.

(ii) *Sodium Salt of Chitin Sulphuric Acid.*—The optimum pH for the production of *N*-acetyl-D-glucosamine was determined by the same method as was used for chitin. Since the sodium salt is soluble in water in the pH range 4-7 the solutions were not shaken but were kept at 37 ± 0.25°C and the reaction was allowed to proceed for 6 days. Excess chitin sulphuric acid was precipitated during the estimations of D-glucosamine and *N*-acetyl-D-glucosamine and to remove the precipitate the solutions were centrifuged after the addition of the colour reagent but before the colour commenced to develop.

(iii) *Chitin Nitrate.*—Repeated experiments showed that the chitinase preparation was without action on finely powdered chitin nitrate.

(*h*) *Isolation of* N-*acetyl-*D-*glucosamine from the Enzymic Degradation of Chitin*

Finely powdered chitin (1.0 g), snail enzyme preparation (50 ml), toluene (3 ml), and sufficient 0.1N aqueous hydrochloric acid to adjust the pH of the solution to 4.8 were thoroughly mixed and kept at 37 ± 0.25°C for 56 days. The solution was shaken vigorously twice each day. Ethanol (350 ml) was added and the precipitated protein and unchanged chitin removed by centrifugation. The clear solution was evaporated to dryness *in vacuo* at 50°C in an inert atmosphere and the residue boiled under reflux with absolute methanol for 90 min to dissolve the *N*-acetyl-D-glucosamine. The hot solution was filtered, concentrated to a small volume (*c*. 10 ml) at 40°C *in vacuo* in an inert atmosphere, and left in the refrigerator for the *N*-acetyl-D-glucosamine to crystallize. A further crop of crystals was obtained on concentrating the mother liquor. Total yield 0.48 g (44 per cent.) m.p. 195°C (decomp.). One crystallization from dry methanol raised the m.p. to 204-205°C (decomp.), undepressed in admixture with an authentic specimen of *N*-acetyl-D-glucosamine, m.p. 204°C (decomp.) (Found: C, 43.4; H, 6.9; N, 6.3%. Calc. for $C_8H_{15}O_6N$: C, 43.4; H, 6.8; N, 6.3%). Colorimetric analysis showed the presence of 4.5 mg D-glucosamine in the solution.

(*i*) *Isolation of* N-*acetyl*-D-*glucosamine from the Enzymic Degradation of the Sodium Salt of Chitin Sulphuric Acid*

The sodium salt of chitin sulphuric acid (1.0 g) was subjected to the same enzymic degradation as was chitin except that it was necessary to add alkali instead of acid to adjust the pH to 4.8. Yield of *N*-acetyl-D-glucosamine 0.33 g, m.p. 190°C (decomp.) raised to 204-205° (decomp.) on crystallization from methanol, undepressed in admixture with an authentic specimen of *N*-acetyl-D-glucosamine, m.p. 204°C (decomp.) (Found: C, 43.5; H, 6.9; N, 6.4%). Colorimetric analysis showed the presence of not more than 5 mg D-glucosamine in the solution.

(*j*) *Acid Hydrolysis of the Sodium Salt of Chitin Sulphuric Acid*

Sodium salt (100 mg) was heated on a steam bath under reflux with 6N aqueous hydrochloric acid (5 ml) for 4 hr. The solution was evaporated to dryness *in vacuo* and the residue dried *in vacuo* over phosphorus pentoxide and potassium hydroxide. The residue was extracted with hot water (0.5 ml), the solution treated with charcoal and filtered. The insoluble material was washed with two lots of water (2 × 0.25 ml). The pH of the solution was adjusted to 5 by the addition of solid sodium bicarbonate, and sodium acetate (200 mg) added. A solution of freshly synthesized (Duff and Bills 1934) 2-hydroxynaphthaldehyde (150 mg) in methanol (7.5 ml) was added and the mixture kept in the dark for 2 hr. The mixture was concentrated to 1 ml *in vacuo* at room temperature and in an inert atmosphere, a few drops of methanol added to the semisolid mass, and the slurry kept at 0°C overnight. The mixture was evaporated to dryness *in vacuo* at room temperature in an inert atmosphere and the residue thoroughly dried *in vacuo* over phosphorus pentoxide and potassium hydroxide. The residue was extracted three times with 10-ml portions of dry chloroform-dry ether (3:1), freed of chloroform and ether, and extracted three times with 1-ml portions of water at 0°C, leaving the water in contact with the solid for some hours each time. Concentration of the aqueous extracts and extraction of the solid which separated with water as before yielded a small additional quantity of yellow solid. Total yield of 2-hydroxynaphthylidine glucosamine 31.3 mg, which after crystallization from methanol had m.p. 200°C (decomp.) undepressed in admixture with an authentic specimen of m.p. 202°C (Found: C, 61.0; H, 5.8; N, 4.2%. Calc. for $C_{17}H_{19}O_6N$: C, 61.2; H, 5.8; N, 4.2%).

Colorimetric analysis of the hydrolysate for D-glucosamine gave 18.1 mg; that isolated in the form of the Schiff base corresponds to 16.8 mg.

(*k*) *Acid Hydrolysis of Chitin Nitrate*

Chitin nitrate (100 mg) was hydrolysed and the Schiff base isolated by the method described for the sodium salt of chitin sulphuric acid. Yield of 2-hydroxynaphthylidene glucosamine 26.4 mg, m.p. 201°C undepressed in admixture with an authentic specimen of m.p. 202°C (Found: C, 61.1; H, 5.8; N,4.1%). Colorimetric analysis of the hydrolysate for D-glucosamine gave 15.2 mg; that isolated in the form of the Schiff base corresponds to 14.2 mg.

(*l*) *Stability of Chitin to Aqueous Hydrochloric Acid at* 100°C

Chitin (18.8 mg) and 2N aqueous hydrochloric acid (10 ml) were heated at 100°C for 7 hr. The mixture was cooled, neutralized, and its volume made up to 50 ml. The solution was centrifuged and 1-ml samples were analysed for D-glucosamine (Found: 1.10 mg glucosamine from 18.8 mg chitin).

(*m*) *Stability of Chitin to Aqueous Alkali at* 100°C

Chitin (2.0 g) and 1N aqueous sodium hydroxide solution (50 ml) were heated on a steam bath for 48 hr, the mixture being shaken at frequent intervals. Saturated aqueous solution of barium hydroxide (20 ml) was added to precipitate any carbonate, the solution kept warm and protected from carbon dioxide for several hours, cooled, and filtered. To test for the complete precipitation of carbonate an additional 1 ml saturated aqueous solution of barium hydroxide was added. Magnesium sulphate heptahydrate (35 g) and concentrated sulphuric acid (2.5 ml) were added and the solution distilled, using a still head incorporating a steam trap. Approximately 50 ml distillate were collected and titrated with 0.1N aqueous sodium hydroxide solution, using phenolphthalein as an indicator (cf. estimation of acetyl groups by Clark 1936, 1937). A control was included which contained no chitin. Difference between the amount of 0.1N alkali required by the control and the chitin solution was 1.25 ml.

III. RESULTS AND DISCUSSION

The difficulty experienced in phosphorylating chitin is in marked contrast to the ease with which sulphuric acid and nitric acid residues can be introduced into the molecule. On the other hand, Karrer, Koenig, and Usteri (1943) were able to phosphorylate chitosan by less drastic methods than those which failed with chitin. These observations, together with the difficulty experienced in degrading chitin with alkali and the report by Schorigin, Makarowa-Semljanskaja, and Anurjewa (1935) that it is exceedingly difficult to methylate chitin by any of the known procedures, place chitin in a class distinct from most other polysaccharides.

From the filter paper partition chromatographic study of the enzymic degradation of chitin by snail (*H. aspersa*) chitinase it is concluded that chitin breaks down to give *N*-acetyl-D-glucosamine together with a trace of D-glucosamine, these being the only degradation products detected. The appearance of D-glucosamine is, perhaps, significant and will be discussed later. Similarly the sodium salt of chitin sulphuric acid is degraded to *N*-acetyl-D-glucosamine and D-glucosamine and again these were the only degradation products detected. On the other hand the snail enzyme preparation appeared to be without action on chitin nitrate.

Graphs illustrating the amounts of *N*-acetyl-D-glucosamine found during the enzymic degradation of chitin and the sodium salt of chitin sulphuric acid at different pH's are given in Figure 1. From these, it can be seen that the chitinase preparation is most active in weakly acid solutions and the pH for optimum activity is 4.8 for both chitin and the sodium salt of chitin sulphuric acid in

McIlvaine's citrate-phosphate buffer, the former at 25°C and the latter at 37°C. During the short period used for these enzymic reactions no D-glucosamine could be detected by the Elson and Morgan (1933) method with chitin as a substrate. When the sodium salt of chitin sulphuric acid was used as the substrate about 0.02 mg D-glucosamine/20 mg substrate was detected, i.e. about 2 per cent. of the estimated amount of *N*-acetyl-D-glucosamine formed.

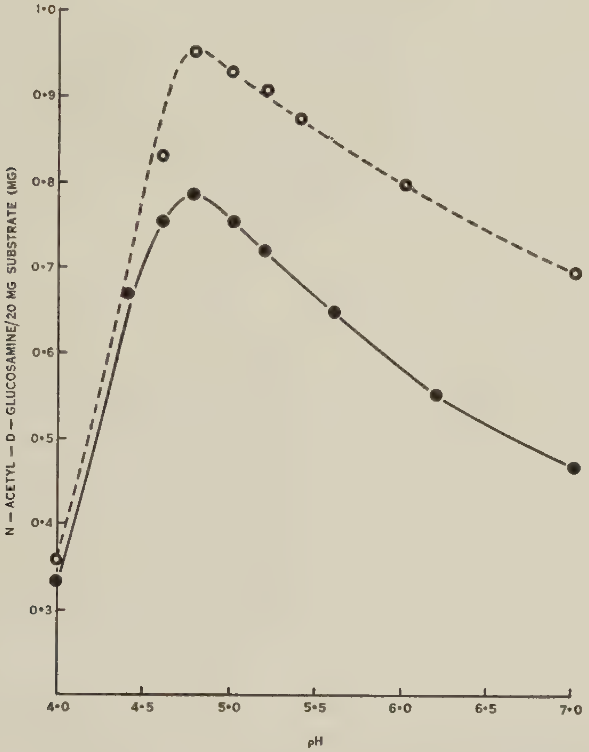

Fig. 1.—Effect of pH on the amount of *N*-acetyl-D-glucosamine formed during the enzymic degradation of chitin (●—●—●) and of the sodium salt of chitin sulphuric acid (O—O—O).

The appearance of D-glucosamine in the enzymic hydrolysate of the sodium salt of chitin sulphuric acid (in excess of that obtained from chitin) indicates that some deacetylation has occurred during the sulphation of chitin. The sulphur content of the sodium salt of chitin sulphuric acid corresponds to approximately 1.4 sulphate groups per *N*-acetyl-D-glucosamine residue. Since only D-glucosamine and *N*-acetyl-D-glucosamine were detected and estimated in the enzymic hydrolysate a sulphatase must be present in the snail enzyme preparation which attacked the *N*-acetyl-D-glucosamine sulphuric acid. The alternative explanation that in the sulphated chitin only about half the *N*-acetyl-D-glucosamine residues carry sulphuric acid groups is not attractive.

That *N*-acetyl-D-glucosamine is in fact the compound formed when snail chitinase reacts with chitin or with the sodium salt of chitin sulphuric acid is shown by the filter paper partition chromatographic studies mentioned above and by isolating 44 per cent. of the theoretical yield of *N*-acetyl-D-glucosamine from chitin and 330 mg from 1 g of the sodium salt of chitin sulphuric acid. Neither of these figures is corrected for unreacted substrate. In both cases D-glucosamine was present in the hydrolysate and it was estimated by the Elson-Morgan method. The D-glucosamine obtained from chitin represented *c.* 1 per cent. of the *N*-acetyl-D-glucosamine isolated and that from the sodium salt of chitin sulphuric acid 1.5 per cent. of the *N*-acetyl-D-glucosamine isolated. Hydrolysis of chitin nitrate or of the sodium salt of chitin sulphuric acid by 6N aqueous hydrochloric acid at 100°C leads to the formation of D-glucosamine, as is shown by the isolation of 2-hydroxynaphthylidene glucosamine. The amount of Schiff base isolated agrees very well with the amount of D-glucosamine estimated to be present. However, comparison of these results with those obtained in the enzymic degradation of the sodium salt of chitin sulphuric acid indicates that considerable decomposition must occur in hot acid solution.

The structure of chitin is a problem which has received a great deal of attention and it is generally accepted that chitin is a polymer built up of *N*-acetyl-D-glucosamine residues. The question as to whether there are any non-acetylated amino groups in chitin is possibly of great biological importance but remains unanswered. Chemists investigating the structure of chitin have invariably used a "purified" chitin, i.e. cuticle which has been repeatedly extracted with hot dilute aqueous alkali or acid, or both. Such samples of chitin give a positive ninhydrin and a positive periodic acid-Schiff (PAS) reaction. This indicates that in the purification process the chitin has undergone some degree of deacetylation. That this is so for chitin treated with hot dilute aqueous hydrochloric acid (cf. Fraenkel and Rudall 1940) is shown by the appearance of D-glucosamine in the aqueous extract and after 7 hr at 100°C 6.6 per cent. conversion of chitin to D-glucosamine occurred. Not only are the amino groups deacetylated but the chitin is hydrolysed. The effect of alkali is more difficult to assess as free D-glucosamine is not stable. However, estimation of the volatile acids formed from purified chitin by the action of 1N aqueous alkali at 100°C for 48 hr indicates that methods of purification involving such alkali treatments remove 1.3 per cent. of the acetyl groups. Again, enzymic degradation of purified chitin produces a small amount of D-glucosamine (cf. Karrer and Hoffmann 1929; Veldkamp 1952) but since the enzymes used cannot be considered pure, this deacetylation could be enzymic. There is little doubt, therefore, that such purified chitin contains free amino groups. The accepted structure of chitin is one which contains no α-glycol groups and no free amino groups and therefore it is expected that chitin would give a negative PAS reaction and a negative ninhydrin reaction. Richards (1952) showed the presence of PAS-positive substances in the epicuticle of many insects and assumed the presence of polysaccharides. However, as mentioned by Brunet (1952), a more reasonable conclusion is that the dihydric phenols known to be present in the epicuticle give the positive PAS reaction. The presence or absence of a positive PAS reaction in various insect procuticles noticed by Richards is more difficult to explain.

The procuticle is considered to be made up of chitin and protein. A positive PAS reaction therefore means that either the chitin contains free amino groups or that some unidentified material reacting positively in the PAS reaction is present. Such unidentified material could be polyphenols, which are being transported through the procuticle, or protein-carbohydrate complexes, which are known to be present in insect cuticles (Hackman 1953; Trim 1941).

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TIME RELATIONS AND THEIR SIGNIFICANCE IN THE OVULATION AND PENETRATION OF EGGS IN RATS AND RABBITS

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Summary

Observations have been made on a total of 577 mated adult rats and 52 mated rabbits.

The mean number of eggs recovered from 362 rats killed between 10.00 a.m. and 5.00 p.m. on the day of ovulation was 9.6 per rat (S.D. 2.2, range 2-20 eggs), or 4.8 per fallopian tube (S.D. 1.9, range 0-13 eggs).

In rats mated under normal colony conditions, ovulation occurred between 12.00 midnight and 4.00 a.m. The ovulation of six eggs in any one rat took about 1 hr. Penetration by sperms occurred 2-4 hr after ovulation. The penetration of all eggs in any one rat took a mean time of 3½ hr. Pronuclei were formed about 3 hr after sperm penetration.

In rats kept under conditions of controlled illumination (light from 3.00 p.m. to 5.00 a.m.; dark from 5.00 a.m. to 3.00 p.m.), ovulation occurred between 11.00 a.m. and 3.00 p.m. The duration of the ovulation period (4 hr) and the time between the beginning of darkness and the commencement of ovulation (about 6 hr) were the same as in the rats mated under normal colony conditions. Similar also were the mean time for ovulation of six eggs in any one rat (¾ hr), the interval between ovulation and sperm penetration (2½-4 hr), the time required for penetration of all eggs in any one rat (3 hr), and the time between the penetration of the eggs and the formation of pronuclei (4 hr). Penetration of all the eggs in any one fallopian tube took about 2 hr. In the course of penetrating the eggs, sperms spent a mean time of ½ hr in the perivitelline space.

Groups of rats were mated at 3.00 a.m. (when ovulation was still in progress), at 7.00 a.m., or at 11.00 a.m. In each instance sperm penetration did not begin until at least 2 hr later. Such a period for the "capacitation" of the sperms is therefore probably normal and does not reflect merely a post-ovulatory alteration in the functional state of the tubes.

In rabbits, sperm penetration began about 10 hr after mating, and all eggs were penetrated by the thirteenth hour. With coitus at the time of induced ovulation, penetrated eggs were not found until 5 hr later, although sperms were present at 4 hr. It appears therefore that capacitation of sperms in the rabbit takes about 5 hr.

The delay of 2-4 hr between ovulation and sperm penetration in normally mated rats suggests that a change, possibly a final stage of maturation, must take place in the egg membranes of the adult rat before sperms can enter the eggs. As no delay was observed in the rabbit, it seems that maturation of the rabbit egg is complete at ovulation.

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I. Introduction

Evidence has been produced which indicates that, in the rat and rabbit, the sperm must spend some time in the female tract, presumably to undergo a process of preparation or "capacitation," before it is capable of penetrating the zona pellucida of the egg (Austin 1951; Chang 1951). This evidence was obtained in experiments that involved manipulation of the sperms and operative interference with the animals—procedures that could be held responsible, in part at least, for some of the effects observed. Confirmatory data were reported by Austin (1952), who found that if rats were permitted to mate after ovulation the eggs were not penetrated until 2 hr or more later, although sperms could be demonstrated at the site of fertilization 1 hr after mating. This delay, together with the rate at which penetration of the eggs then proceeded, seemed to point unequivocally to a need for capacitation of the sperms. The avoidance of operative interference in the experiment lent force to the conclusions drawn, but the objection could be raised that the mating of the rats had taken place several hours after ovulation, whereas under normal circumstances mating occurs about 10 hr before ovulation (Boling *et al.* 1941). It was therefore possible that the delay observed by Austin (1952) may only have been a feature of the abnormal time relations and have indicated a deterioration of the conditions within the fallopian tube that are required for normal fertilization.

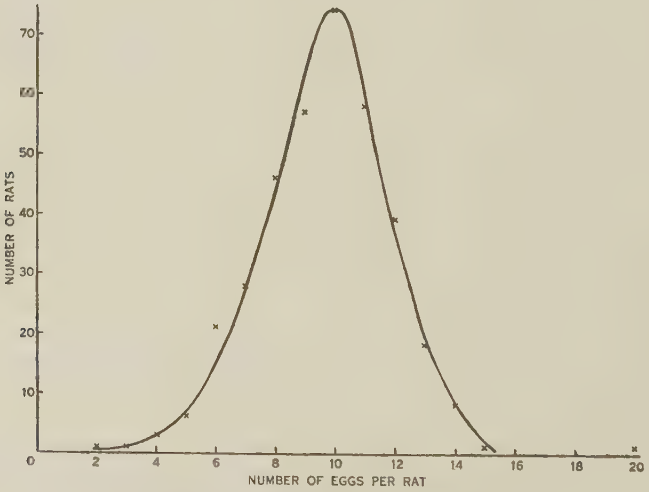

Fig. 1.—Frequency distribution of eggs in 362 rats.

In the present paper these problems receive further attention, and supporting evidence is brought forward to show that a period for capacitation is a normal preliminary to sperm penetration. During the course of investigations, data were obtained on several different aspects of the time relations of ovulation and sperm entry. As this appeared to be a promising field of enquiry, the range of the observations was extended and the material thus gained forms the basis for the greater part of this paper.

II. METHODS

The rats used were of both albino and "hooded" strains, and were all adult animals, 3 months of age or more. The rabbits were crossbreds, aged between 6 and 15 months.

The time of ovulation was based upon the finding of eggs in the fallopian tubes. Eggs were recovered by dissecting the tubes under normal saline solution and were examined in the fresh state with a phase-contrast microscope, as described by Austin and Smiles (1948).

Ovulation was induced in the rabbit by the intravenous injection of 40 I.U. of chorionic gonadotrophin ("Gonan," B.D.H.).

Some of the rats were kept in a specially constructed cabinet in which the light was controlled. The only light was provided by 25-watt tungsten-filament lamps, which were switched on at 3.00 p.m. and off at 5.00 a.m. each day by means of an electric clock relay. The rats were kept under these conditions during the period of observations and for 3-4 wk beforehand. The practicability of controlling oestrous phenomena in the rat by artificially changing the times of light and dark was first clearly demonstrated by Hemmingsen and Krarup (1937).

III. OBSERVATIONS

(*a*) *Number of Eggs Recovered from Adult Rats*

To determine the number of eggs produced at spontaneous ovulation in mated adult rats, data were collected from a total of 362 animals that had been kept under normal colony conditions and killed at different times between 10.00 a.m. and 5.00 p.m. on the day of ovulation.

The frequency distribution of the numbers of eggs is illustrated in Figure 1. The total number of eggs counted was 3477, and the mean was found to be 9.6 eggs per rat, with a standard deviation of 2.2 and a range of 2-20 eggs. In 94 per cent. of the rats, the number of eggs per rat ranged from six to 13. The frequency distribution of the number of eggs per fallopian tube was similar in shape. The mean number of eggs was 4.8, standard deviation 1.9, and range 0-13 eggs.

(*b*) *Time Relations of Ovulation and Sperm Penetration in Rats*

(i) *Under Normal Colony Conditions.*—To determine the times of ovulation and sperm penetration in animals kept under normal colony conditions, female rats were placed with males during the afternoon and those found to have copulation plugs when examined at 11.00 p.m. were put aside. Groups of these animals were killed at hourly intervals from midnight to 5.00 a.m. and at 7.30 a.m. The fallopian tubes were dissected to determine whether eggs were present, and the eggs were examined for evidence of sperm penetration. Observations were based on a total of 115 mated rats (Table 1).

At midnight, eggs were found in only two out of 15 rats and these provided a total of three eggs, none of which contained sperms. At 1.00 a.m., a higher proportion of the rats (12 out of 30) yielded eggs, one of which showed sperm

Table 1

TOTAL NUMBERS OF EGGS AND NUMBER OF PENETRATED EGGS IN RATS MATED UNDER NORMAL COLONY CONDITIONS AND KILLED ABOUT 5½-13 HR AFTER SUNSET

Time of Killing	No. of Rats Killed		No. of Eggs Recovered		Mean No. of Eggs per Rat Killed		Eggs Penetrated (%)
	Total	With Eggs	Total	Penetrated	Total	Penetrated	
Midnight	15	2	3	0	0·2	0	0
1.00 a.m.	30	12	57	1	1·9	0·03	1·6
2.00 a.m.	20	13	89	5	4·5	0·25	5·6
3.00 a.m.	15	12	95	13	6·3	0·9	13·8
4.00 a.m.	11	11	104	27	9·5	2·5	26·3
5.00 a.m.	10	10	84	34	8·4	3·4	40·5
7.30 a.m.	14	14	138	124	9·9	7·9	89·8

penetration. From then on, progressively more rats were found to have eggs and an increasingly larger proportion of the eggs had been penetrated by sperms. Ovulation was completed in the rats as a group by about 4.00 a.m. On the other hand, the incidence of penetrated eggs continued to rise for the rest of the period of observations, although most of the eggs were penetrated by 7.30 a.m.

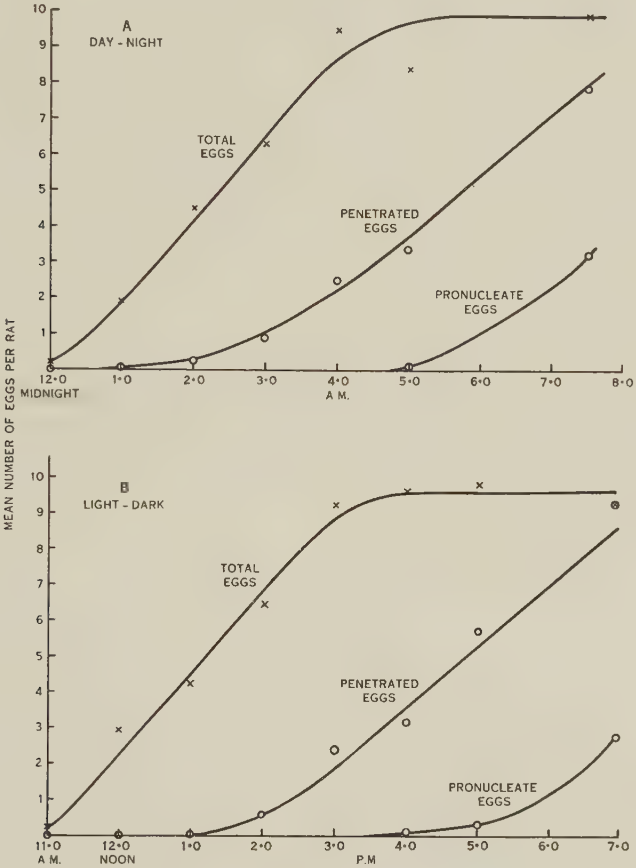

Fig. 2.—Progressive changes in the mean numbers per rat of total eggs, penetrated eggs, and eggs with pronuclei. *A*, rats kept under natural illumination; sunrise was between 4.30 and 5.00 a.m. and sunset between 6.00 and 7.00 p.m. *B*, rats kept under controlled artificial illumination; light was turned on at 3.00 p.m. and off at 5.00 a.m.

The number of eggs recovered at each hour (mean for all rats killed) was 0.2 at midnight, and increased progressively to a mean of 9.5 per rat at 4.00 a.m. (Table 1). Thereafter there was no significant change. The trend of the results is illustrated by the curve for "total eggs" in Figure 2*A*. It is apparent that, for the rats as a group, ovulation took about 4 hr, and was confined almost entirely to the period between midnight and 4.00 a.m. This work was done during November and December when sunrise was between 4.30 and 5.00 a.m. and sunset between 6.00 and 7.00 p.m.; the days thus varied from 13 to 14½ hr in length.

An approximate assessment of the time required for ovulation to occur in any one rat can also be made. From the data on the number of eggs recovered (Section III (*a*)), it can be shown that 97 per cent. of rats produced six or more eggs. When the curve representing the proportion of rats with eggs was compared with that for the proportion of rats with six or more eggs, an interval corresponding to about 1 hr was observed between them. This is interpreted to signify that in any one rat a mean time of about 1 hr was required for six eggs to gather in the fallopian tubes. Similarly, the mean time required for three eggs to arrive in any one fallopian tube was found to be about ½ hr.

A measure of the rate of penetration of the eggs by sperms is obtained by determining the mean number of penetrated eggs per rat killed, at each interval; this was zero at midnight and 0.03 eggs per rat at 1.00 a.m., and increased to 7.9 eggs per rat at 7.30 a.m. (Table 1). These results form the data for the curve marked "penetrated eggs" in Figure 2*A*. It appears that the eggs are penetrated at a rate that is a little slower than the rate at which they are ovulated. There is an interval corresponding to 2-4 hr between the curves for the mean number of eggs per rat and the mean number of penetrated eggs per rat. Evidently penetration of the eggs in the rats as a group occurs about 2-4 hr after ovulation, the interval being slightly longer for the later than for the earlier times of penetration.

The number of penetrated eggs may also be expressed as a percentage of the total eggs obtained, viz. 1.6 per cent. at 1.00 a.m., increasing to 89.8 per cent. at 7.30 a.m. (Table 1). These results are shown as curve *N* in Figure 3. It can be seen that the increase in the proportion of penetrated eggs is very slow initially, but becomes rapid after 4.00 a.m.

As records were kept for individual rats, it was possible to calculate the proportion of rats with one or more penetrated eggs as well as the proportion of rats in which all eggs were found to contain sperms. Thus, at 1.00 a.m., 2.00 a.m., 3.00 a.m., and 4.00 a.m., the number of rats found with penetrated eggs was one (3.3 per cent.), four (20 per cent.), four (27 per cent.), and seven (64 per cent.) respectively. In none of these rats, however, were all eggs penetrated. Among the animals killed at 5.00 a.m., nine (90 per cent.) were found with penetrated eggs and in one rat (10 per cent.) all the eggs contained sperms. At 7.30 a.m. all of the 14 rats killed carried penetrated eggs (100 per cent.) and in nine rats (64 per cent.) all the eggs were penetrated. These results are shown as curves N_1 and N_2 in Figure 4. The curves are separated by an interval which corresponds to about 3½ hr. This is interpreted as indicating that penetration of all the eggs in any one rat after normal mating takes about 3½ hr from the penetration of the first egg.

Pronuclei were observed in one egg (0.1 eggs per rat) from the rats killed at 5.00 a.m., and in 45 eggs (3.2 per rat) from the rats killed at 7.30 a.m. These results are represented by the curve marked "pronucleate eggs" in Figure

2*A*. It would appear that the pronuclei were formed at a mean interval of about 3 hr after penetration of the eggs.

(ii) *After Delayed Mating.*—The rate of penetration of eggs after delayed mating was studied in a total of 259 mated rats. Rats were selected for oestrus by the nature of the vaginal smear and placed with males for 10-15 min from 3.00 to 3.30 a.m., from 7.00 to 7.30 a.m., or from 11.00 to 11.30 a.m. Coitus was nearly always seen to occur immediately the animals were put together. For reference purposes the times of mating are stated as 3.00 a.m., 7.00 a.m., and 11.00 a.m. respectively.

Forty-five rats that mated at 3.00 a.m. were killed 2 (15 rats), 3 (10 rats), 5 (10 rats), and 7 hr (10 rats) after mating. The numbers of penetrated eggs recovered were none, 25 (28 per cent.), 66 (73 per cent.), and 88 (100 per cent.) respectively. These results are the data from which curve 1 in Figure 3 is drawn.

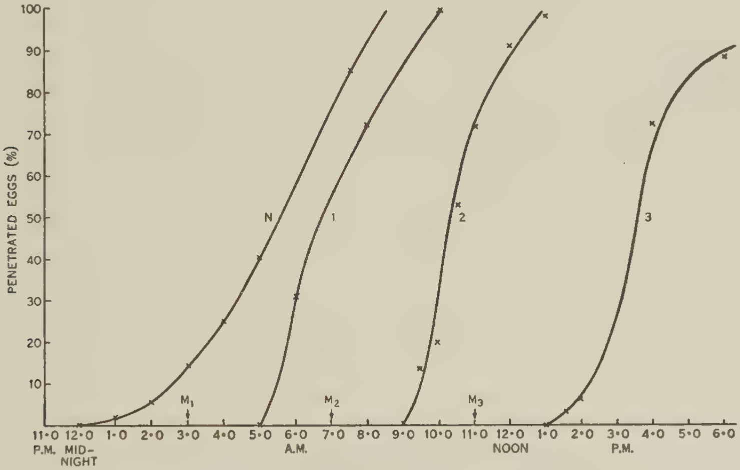

Fig. 3.—Percentages of penetrated eggs in rats at various hours after coitus. *N*, normal mating. 1, 2, and 3, mating at 3.00 a.m., 7.00 a.m., and 11.00 a.m. respectively (M_1, M_2, and M_3).

The numbers of penetrated eggs obtained from 65 rats mated at 7.00 a.m. have been reported in an earlier paper (Austin 1952), but since then some further observations have been added. Twelve more rats have been killed at 2½ hr, 66 at 3 hr, and 31 at 3½ hr after coitus, making a total of 22 rats for 2½ hr, 76 for 3 hr, and 31 for 3½ hr. The total number of eggs recovered is now 221, 752, and 303, and the number of penetrated eggs 14, 154, and 162 respectively. All this material forms the basis for curve 2 in Figure 3.

The 50 rats that were not permitted coitus until 11.00 a.m. were killed 2, 2½, 3, 5, or 7 hr after mating. The numbers of penetrated eggs were none, five (4 per cent.), seven (7 per cent.), 64 (67 per cent.), and 81 (89 per cent.) respectively; these are represented by curve 3 in Figure 3.

Curves 1, 2, and 3 show clearly that penetration of the eggs commences only after about 2 hr after mating. The delay is of the same duration in each instance although the hour of mating varied from 3.00 to 11.00 a.m. (as indicated by the letters M_1, M_2, and M_3). When penetration of the eggs does begin, it proceeds initially much more rapidly than that observed after normal mating (curve N).

Observations on the rats mated at 7.00 a.m. included both the total number of eggs and the number of penetrated eggs in individual rats, and from these data the proportion of rats with one or more penetrated eggs and of rats with all eggs penetrated has been calculated. At 1 hr after mating, no rat carried penetrated eggs; at 2 hr and 2½ hr one rat (7 per cent.) and 10 rats (46 per cent.) respectively carried penetrated eggs, but in no rat were all eggs penetrated. Among the rats killed at 3, 3½, 4, 5, and 6 hr after coitus, 39 (51 per cent.), 26 (84 per cent.), nine (90 per cent.), 10 (100 per cent.), and 10 (100 per cent.) respectively had one or more eggs penetrated by sperms. At the same hours, the numbers of rats in which all eggs were penetrated were one (1.5 per cent.), seven (23 per cent.), four (40 per cent.), six (60 per cent.), and eight (80 per cent.) respectively. Curves D_1 and D_2 in Figure 4 are based upon this information. The curves are separated by an interval corresponding to 1-2 hr, and it is concluded that, once the penetration of the eggs had begun, the mean time required for penetration of all the eggs in any one rat was about 1-2 hr. In the same way, it can be shown that the mean time required for penetration of all the eggs in any one fallopian tube was about 1 hr.

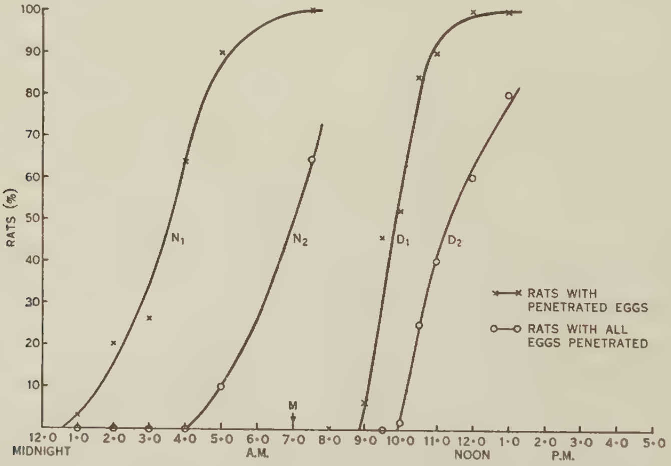

Fig. 4.—Percentage of rats with penetrated eggs (N_1 and D_1) compared with the percentage of rats with all eggs penetrated (N_2 and D_2) at various hours after normal matings (N_1 and N_2) and after mating at 7.00 a.m. (D_1 and D_2).

(iii) *Under Conditions of Controlled Illumination.*—Adult female rats kept with males under conditions of controlled illumination in the special cabinet were examined each morning for signs of mating, and rats with copulation plugs were killed at each hour from 11.00 a.m. to 5.00 p.m. and at 7.00 p.m. The eggs were recovered and studied in the manner already described. Results were obtained from a total of 100 animals (Table 2).

Of the rats killed at 11.00 a.m. only one had eggs (two eggs), and of those killed at noon and 1.00 p.m. five and 12 rats yielded 29 and 88 eggs respectively. None of these eggs was penetrated by sperms. At 2.00 p.m. 12 rats had a total of 97 eggs, of which seven were penetrated by sperms. Thereafter all rats yielded eggs, and the proportion of penetrated eggs increased progressively. When the results are expressed as the mean number of eggs per rat killed, the values increase steadily from 0.2 at 11.00 a.m. to 9.2 at 3.00 p.m. After this the figures show no significant change. The mean number of penetrated eggs per rat killed is zero for 11.00 a.m., 12.00 noon, and 1.00 p.m., and 0.5 for 2.00 p.m.; for the subsequent hours there is a progressive increase in the values, which reach 9.2 penetrated eggs per rat at 7.00 p.m. These results are illustrated by the curves in Figure 2*B*. For the rats as a group, ovulation apparently took about 4 hr and occurred almost entirely in the period 11.00 a.m. to 3.00 p.m. The time taken is very similar to that observed in the rats mated under normal colony conditions. An estimate of the mean time required for six eggs to gather in the fallopian tubes of any one rat is about ¾ hr, and for three eggs in any one fallopian tube ½ hr. These results are also similar to those obtained from the rats mated under normal colony conditions (Section III (*b*) (i)).

The interval between the curves for total eggs and penetrated eggs in Figure 2*B* corresponds to a period of about 2½-4 hr. This estimate for the time between ovulation and sperm penetration in the rats as a group is virtually the same as the 2-4 hr observed for the rats mated under normal colony conditions.

The number of penetrated eggs, expressed as a percentage of the total eggs recovered, was zero for 11.00 a.m., 12.00 noon, and 1.00 p.m., and 7.2 per cent. for 2.00 p.m., and then increased to reach 100 per cent. at 7.00 p.m. (Table 2). The results show a trend similar to that shown by the corresponding data for the rats mated under normal colony conditions (see curve *N* in Fig. 3).

Comparison of the curves representing the proportion of rats with one or more penetrated eggs and the proportion with all eggs penetrated led to a similar conclusion to that drawn from the data for the rats mated under normal colony conditions: once penetration had begun, the mean time required for penetration of all the eggs in any one rat was about 3 hr. By the same method it was shown that about 2 hr was needed for all the eggs in any one fallopian tube to be penetrated by sperms.

Among the penetrated eggs there were some in which sperms had penetrated the zona pellucida but had not yet entered the vitellus and so lay in the perivitelline space. The number of these eggs and the proportion they represented of the total eggs recovered were as follows: at 11.00 a.m., 12.00 noon, and 1.00 p.m., zero; at 2.00 p.m., four (4.1 per cent.); at 3.00 p.m., eight (6.2 per cent.); at 4.00 p.m., nine (9.4 per cent.); at 5.00 p.m., 10 (10.1 per cent.); and at 7.00 p.m., one (1.0 per cent.).

As all but one of the eggs examined at 7.00 p.m. contained sperms in the vitellus, it may reasonably be assumed that eggs seen earlier with sperms only

Table 2

TOTAL NUMBERS OF EGGS AND NUMBER OF PENETRATED EGGS IN RATS MATED UNDER CONDITIONS OF CONTROLLED ILLUMINATION AND KILLED 6-14 HR AFTER THE ONSET OF DARKNESS

Time of Killing	No. of Rats Killed		No. of Eggs Recovered		Mean No. of Eggs per Rat Killed		Eggs Penetrated (%)
	Total	With Eggs	Total	Penetrated	Total	Penetrated	
11.00 a.m.	10	1	2	0	0·2	0	0
Noon	10	5	29	0	2·9	0	0
1.00 p.m.	20	12	88	0	4·4	0	0
2.00 p.m.	15	12	97	7	6·5	0·5	7·2
3.00 p.m.	14	14	129	37	9·2	2·6	28·7
4.00 p.m.	10	10	96	31	9·6	3·1	32·3
5.00 p.m.	10	10	99	59	9·9	5·9	59·5
7.00 p.m.	11	11	101	101	9·2	9·2	100

in the perivitelline space would all eventually have sperms in the vitellus. During the period 2.00-5.00 p.m. the rate of sperm penetration was approximately linear (Fig. 2*B*), so that the frequency with which eggs were seen with sperms in the perivitelline space only should be proportional to the time the sperms spent in the perivitelline space. For the same period, an estimate of the proportion of eggs with sperms in the perivitelline space only is given by the mean of the figures for 3.00, 4.00, and 5.00 p.m., namely 8.6 per cent. The average time (t) spent by the sperm in the perivitelline space may then be calculated by the formula

$$t = \frac{8.6 \times T}{P - p},$$

where T is the period covered by the observations (3 hr), and P and p are the percentages of eggs penetrated by sperms at 5.00 p.m. and 2.00 p.m. respectively (Table 2). The result obtained was 29.6 min, or about ½ hr.

Among the penetrated eggs from the rats killed at 4.00 p.m., one egg (0.1 per rat), and at 5.00 p.m. two eggs (0.2 per rat), had early forms of pronuclei; at 7.00 p.m. 27 eggs (2.5 per rat) had pronuclei in the early and intermediate stages of development. By comparing the curve based upon these figures with that for the mean number of penetrated eggs per rat (Fig. 2*B*), it is seen that a mean period of about 4 hr from penetration was required for the formation of a pronucleus. This is reasonably similar to the estimate based upon the data from rats mated under normal colony conditions (3 hr).

(*c*) *Time Relations of Sperm Penetration in Rabbits*

Observations were made on 14 rabbits mated in the normal manner and killed 10-16 hr later. The eggs were recovered, fixed, embedded in paraffin, and cut in sections 7 μ thick. After staining by the Feulgen technique the preparations were examined to determine whether sperm penetration had occurred. Four rabbits were killed 10 hr after mating and yielded a total of 19 eggs; nine eggs were successfully brought through the histological procedures and five (56 per cent.) of these were found to contain sperms. From four rabbits killed 13 hr after mating, 30 eggs were recovered, and 19 were examined. All these were found to contain sperms. Similarly, all of the 28 eggs examined out of a total of 48 eggs obtained from six rabbits killed 16 hr after coitus showed evidence of sperm penetration (Table 3).

In a second experimental series, 38 rabbits received an intravenous injection of chorionic gonadotrophin for the induction of ovulation and were permitted coitus 10 hr later, i.e. at about the time of ovulation. The eggs from all these rabbits except the last group were examined histologically in the manner just described (Table 3). At 4 hr after mating, none of the 19 eggs examined had been penetrated by sperms. The rabbits killed 5 hr after mating yielded 39 eggs; four of the 26 eggs examined contained sperms. The corresponding figures for the rabbits killed 6 and 8-10 hr after mating were respectively: 27 and 17 eggs examined, and nine eggs (33 per cent.) and 12 eggs (71 per cent.) found to contain sperms. The last group, of 17 rabbits, was killed 15-20 hr after mating. The eggs were all examined in the fresh state with the phase-contrast microscope; 82 eggs (73 per cent.) showed evidence of sperm penetration. The percentages for the rabbits killed at 8-10 hr and at 15-20 hr did not differ significantly, and it would seem that, when mating occurs at about the time of ovulation, not more than about three-quarters of the eggs can be expected to undergo fertilization.

IV. DISCUSSION

The time of ovulation observed in this laboratory for a group of 115 rats kept and mated under normal colony conditions was between 12.00 midnight and 4.00 a.m. Similar results were obtained by Everett (1948), who killed 28 rats between 12.45 and 4.00 a.m., and noted that ovulation was in progress as early as 1.00 a.m. and was completed (eight or more tubal eggs) in all rats examined after 2.30 a.m. The narrower range observed by this author may have been owing to the smaller number of experimental animals or to his use of a genetically more homogeneous group of rats. Consistent also is the statement by Pederson (1951) that ovulation in his rats was most likely to occur at 3.00 a.m.

TABLE 3

PROPORTION OF EGGS SHOWING SPERM PENETRATION IN RABBITS KILLED AT VARIOUS TIMES AFTER NORMAL MATING AND AFTER MATING AT THE TIME OF INDUCED OVULATION

Mating	No. of Rabbits	Time after Mating (hr)	Total Eggs Recovered	Eggs Examined	Penetrated Eggs	
					No.	Per Cent.
Normal	4	10	19	9	5	56
	4	13	30	19	19	100
	6	16	48	28	28	100
At time of ovulation	6	4	50	19	0	0
	6	5	39	26	4	15
	5	6	36	27	9	33
	4	8-10	44	17	12	71
	17	15-20	112	112	82	73

The occurrence of ovulation close to midnight has also been noted in other rodents: about 1.00 a.m. for the hamster (Ward 1946), and between 11.30 p.m. and 1.30 a.m. for the mouse (Runner 1947).

In the present series of experiments the rate of ovulation, and the relationship between ovulation and the times of light and dark, have been found to be very similar whether the animals were kept under natural lighting or in a cabinet under controlled artificial illumination. Ovulation began in the rats as a group about 6 hr after the commencement of darkness and required the last 4 hr of darkness to reach completion. The experiments involving natural lighting were made during November and December when the night lasted about 10 hr; the time relationship of ovulation must necessarily vary at other seasons of the year when the nights are either longer or shorter than 10 hr. A close adherence of ovulation to the time relations of artificial "day" (4.00 a.m. to 7.15 p.m.) and "night" was also reported by Snell *et al.* (1940) for mice kept under controlled artificial illumination. They noted that ovulation in mated mice most commonly occurred between "midnight" (i.e. 4¾ hr after the beginning of darkness) and 2.00 or 3.00 "a.m.," but was sometimes as late as 4.40 "a.m." (i.e. 40 min after the end of darkness).

As already mentioned, the occurrence of ovulation is inferred from the finding of eggs in the fallopian tube. Whether the eggs leave the ovary with the same frequency with which they reach the tube is unknown, but is presumed to be so. The impression gained is that the eggs, singly or in pairs, pass rapidly through the first part of the ampulla, to gather in the distended region, where they form the characteristic coherent group. It is therefore considered that an indication of the time required for ovulation in any one rat can be obtained from a comparison of the curves based upon the proportion of rats with eggs, and of rats with six or more eggs, in the fallopian tubes. The figure of six was chosen because the great majority (97 per cent.) of rats produce six or more eggs. The estimates made, both for the rats kept under natural lighting and for those under controlled artificial illumination, were similar, and indicate a period of about ¾-1 hr. This is much shorter than the time required for the rats as a group to produce a mean of six or more eggs, namely 2½-3 hr (from Fig. 2); presumably the longer period for the group is due mainly to variations in the times at which ovulation begins in individual rats. Reliable estimates could not be made for the production of numbers of eggs much in excess of six, because the higher the number the lower the proportion of rats represented and the greater therefore the error in calculation. It is of course likely that, for the ovulation of more than six eggs, proportionately longer periods are involved.

The time between ovulation and sperm penetration, under both natural and artificial lighting conditions, has clearly been shown in the present investigation to be about 2-4 hr. This appears at first to conflict with the statement by Odor and Blandau (1951) that in their experiments 47 per cent. of eggs were found to have been penetrated during the first hour after ovulation, 54 per cent. during the second hour, and 90 per cent. during the third hour. However, Odor and Blandau did not determine ovulation time directly but depended upon the generalization made earlier, that ovulation is proceeding or has been completed in most rats 10 hr after they show a specified copulatory response to handling (Boling *et al.* 1941). Now Boling *et al.* also found that ovulation began in the rats as a group about 6½-7½ hr after the response, so that Odor and Blandau's (1951) "first hour after ovulation" is therefore about the fourth hour after the commencement of ovulation in the rats as a group. Thus Odor and Blandau do not seem to have interpreted their findings quite correctly, but it appears probable that, had their data been adequate, these authors would also have found an interval of about 2-4 hr between ovulation and sperm penetration.

In an earlier paper (Austin 1952) it was reported that rats mated after ovulation, at 7.00 a.m., did not yield penetrated eggs until at least 2 hr later. This was put forward as further evidence in support of the idea that the sperm requires to spend a period for "capacitation" in the female tract before it can penetrate the egg. More data have now been obtained from rats mated at 7.00 a.m. and these are consistent with the earlier results. It has also been shown that the same interval of at least 2 hr is evident whether the rats are mated at 3.00 a.m., when ovulation is still in progress, or at 11.00 a.m., which is 7-11 hr after ovulation. In each group the numbers of sperms at the site of fertilization have been counted (Braden and Austin 1954). The numbers are small at 1 hr and increase during the first 2½-4 hr after mating. On these grounds the suggestion may be made that it is the changes in sperm concentration that are responsible for the interval of 2 hr between mating and sperm penetration. The inadequacy of this theory, however, is revealed upon closer study of the data, particularly the regular nature of the rise shown by sperm numbers during the first 2 or 3 hr, and by contrast, the rapidity with which the proportion of penetrated eggs increases for most of the period of sperm penetration. Consideration of these functions leads plainly to the expectation that 2 hr after coitus the proportion of penetrated eggs should be of the order of 10-20 per cent., whereas the observed incidence is less than 1 per cent. There seems to be no doubt therefore that the interval of about 2 hr can be imputed to the need for the sperm to spend some time in the female tract before it can penetrate the egg. The fact that the interval was much the same whether mating occurred during or after ovulation is interpreted as an indication that the same period for capacitation would be required when mating occurs before ovulation, as under normal circumstances.

In the rabbit, sperm penetration evidently commences very shortly after ovulation, for about half the eggs recovered 10 hr after mating were found to be penetrated. That ovulation has in fact not yet been completed at this hour is evident from the relatively low mean of 4.8 eggs per rabbit; at later hours the means varied from 6.5 to 11.0 eggs per rabbit. At 13 and 16 hr after mating, all eggs were penetrated. Data described elsewhere (Braden, Austin, and David 1954) indicate that penetration of the eggs is probably completed well under 13 hr after mating, for the eggs by that time were found to contain a mean of about 14 sperms per egg. In contrast to these results, the penetration of eggs from rabbits mated at about the time of artificially induced ovulation does not begin until 5 hr after mating and then increases only slowly to reach a maximum about 8-10 hr after mating. In separate communications, it is shown that sperms are present at the site of fertilization 4 hr after coitus, both with normal mating (Braden 1953) and with delayed mating (Braden and Austin 1954). Although more sperms were present 10-12 hr after coitus with normal mating than 4-6 hr after coitus with delayed mating, the difference was insufficient to explain either the absence of penetrated eggs at 4 hr with delayed mating or the much slower rate at which penetration later proceeds. The data therefore add further support to the theory that, in the rabbit also, the sperm requires to spend a period within the female genital tract before it can penetrate the egg (Austin

1951; Chang 1951). The duration of the period appears to be at least 5 hr, more than twice the minimum time required by the rat sperm (2 hr).

Because the interval between coitus and sperm penetration in rats and rabbits, mated during or after ovulation, has been put forward as evidence for the capacitation of the sperm, it may be argued that the same interpretation should be placed upon the interval that occurs between ovulation and sperm penetration in normally mated rats. As the sperms from a normal mating spend 9 or 10 hr in the female tract before ovulation, this suggestion would imply that capacitation involves an interaction between the sperm and the egg, or between the sperm and the liquor folliculi which reaches the tube with the egg. Against such a theory are the observations that sperm penetration can occur within the first hour after ovulation when this is induced artificially in immature rats (Austin 1951) and that sperm penetration into rabbit eggs normally takes place within an hour of ovulation. Furthermore, in the rabbit, capacitation may occur in the uterus (Chang 1951). It seems more probable that during the 2-4 hr interval some change occurs in the egg membranes that must be completed before sperms can enter the egg. The change may represent a final stage in the process of maturation, and may well be more rapid in the immature rat, under the influence of gonadotrophic hormones, than in the adult rat after spontaneous ovulation. The need for such a change would account for the slow initial increase in the percentage of penetrated eggs in the normally mated rats (Fig. 3, curve *N*). In the same way, the occurrence of the change probably underlies the shorter time required for penetration of all the eggs in any one rat, observed after mating at 7.00 a.m. as compared with normal mating (Fig. 4).

Since the number of sperms about the eggs during the period when penetration is taking place is much the same with both these mating procedures (Braden and Austin 1954), the difference between the times required for penetration could be attributed to the fact that by 7.00 a.m. all eggs would have completed their maturation. The eggs would then be penetrable to the first sperm that approached them provided it had undergone capacitation.

V. Acknowledgment

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INDUCTION AND INHIBITION OF THE SECOND POLAR DIVISION IN THE RAT EGG AND SUBSEQUENT FERTILIZATION

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Summary

Among 227 eggs from 23 untreated oestrous rats, the first polar body had persisted in three eggs (1.3 per cent.) and the second polar division had occurred spontaneously in two eggs (0.9 per cent.).

Emission of the second polar body was induced, in about an hour, by subjecting the rat to anaesthesia with ether, chloroform, ethyl chloride, ethyl alcohol, paraldehyde, nitrous oxide, or intraperitoneal "Nembutal." Ether and nitrous oxide influenced the most eggs. "Nembutal," administered subcutaneously, caused no polar body formation. The effect of anaesthetics is probably related to the production of cellular anoxia, and not to the fall in body temperature, for both ether and subcutaneous "Nembutal" depressed the body temperature to the same extent.

Cold-shock treatment (ice) led to the formation of the second polar body in virtually all the eggs, and hot-shock treatment (45°C) in none of them.

In untreated rats, the changes undergone by the male and female elements were closely correlated during the early stages of fertilization. A coordinating influence was also evident in the restoration of the normal relationship after it had been disturbed by experimental treatment.

Shrinkage of the vitellus, involving a reduction of 13 per cent. in volume, was found after sperm penetration, and sometimes after cold-shock treatment.

Sperms can readily penetrate into eggs that have emitted the second polar body under artificial stimulation; the block to polyspermy is unaffected and seemingly normal fertilization and cleavage may follow.

Resumption of the second polar meiosis was inhibited by treatment with hot shock or with colchicine. The influence of hot shock was transient whereas that of colchicine was more lasting. A high incidence of polyspermy (16 per cent.) was observed after hot-shock treatment, due evidently to interference with the block to polyspermy.

The ways in which the experimental treatments could induce heteroploidy in rat embryos are discussed.

The development of the block to polyspermy, the shrinkage of the vitellus, and the emission of the second polar body are seen as independent processes, capable of being evoked separately. Only the block to polyspermy appears to be a specific response to sperm penetration.

I. INTRODUCTION

In the eggs of mammals, as in those of many invertebrates and lower vertebrates, the immediate consequences of sperm penetration include the development of a block to polyspermy, the shrinkage of the vitellus, and the resumption

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of the second maturation division leading to the abstriction of the second polar body. These reactions are held to indicate the occurrence of "activation," or the arousal of the egg from a dormant state. Normally, these changes also mean that the entry of further sperms is precluded and that the chromosome number is reduced to the haploid state, preparatory to syngamy. In the absence of sperm penetration, the animal egg may yet be induced to undergo activation by artificial means, when the same changes may also occur and may even lead to parthenogenetic development, although variations occur between species and according to the treatment used.

Among mammalian eggs those of the rabbit have been chiefly used for studies on artificial activation and it has been shown that several of the agents known to be effective on the eggs of lower animals also induce activation in the rabbit egg when applied *in vitro* (Pincus and Enzmann 1935, 1936; Pincus 1936, 1939; Pincus and Shapiro 1940; Thibault 1947*a*, 1948, 1949). The agents include hyper- and hypotonic solutions, butyric acid, heat, and cold. Cold treatment was found to be effective *in vivo*, when ice was applied to the fallopian tubes shortly after ovulation. The activated rabbit egg generally showed a shrinkage of the vitellus, but the abstriction of the polar body was not common, the spindle giving place instead to a single (diploid) or two (presumably haploid) nuclei. Regular cleavage often followed activation, but Thibault (1949) considered that only eggs which form diploid nuclei could undergo such parthenogenetic development. Thibault (1947*b*, 1949) and Thibault and Ortavant (1949) also studied the reactions of sheep and rat eggs to cold shock. They found that the sheep egg behaved similarly to the rabbit egg, but that the rat egg differed, for it regularly extruded the second polar body and was never seen with reformed nuclei or undergoing cleavage. Thibault (1949) observed that, in the rat, ether anaesthesia or barbiturate narcosis, without other treatment, would induce the activation of a proportion of the eggs in the intact animal. He ascribed this effect partly to a direct action of ether on the eggs and partly to the drop in body temperature that occurred during anaesthesia or narcosis.

In the eggs of many of the lower animals, concomitantly with the shrinkage of the vitellus, a fertilization membrane is elevated after sperm penetration and this is not penetrable to sperms. The elevation of the membrane also constitutes one of the earliest and surest criteria of activation when this is provoked by artificial means. Consequently, eggs that are artificially activated are rendered incapable of normal fertilization. A fertilization membrane has not been described in the mammalian egg, except for one possible instance in *Cricetus* (Venable 1946), but shrinkage of the vitellus commonly occurs, and it may be argued with Thibault (1949) that artificially activated mammalian eggs are unfertilizable. This effect, however, does not appear to have been demonstrated experimentally.

The investigations to be described in the present paper were made in order to obtain further information on the activation of rat eggs by sperms, anaesthetics, and other agents, to determine whether eggs could be penetrated by sperms after artificial activation, and, should this be possible, to study the cytology of fertilization in these eggs.

II. Methods

Oestrous rats were selected between 8.00 and 9.00 a.m. by inspection of the vaginal smear. If required for studies on fertilization, the rats were placed with males for ½ hr between 9.00 and 10.00 a.m., and those that mated were detected by the presence of copulation plugs. It has previously been shown that oestrous rats in this colony have generally completed ovulation by 7.00 a.m. and that, if they then copulate, their eggs will be fertilized after an interval of at least 2 hr (Austin 1952). Under these circumstances, it is possible to subject the eggs to experimental treatment after mating and ovulation but before the time when fertilization would begin. Treatments were therefore applied between 10.00 and 11.00 a.m. The rats were killed the same afternoon, or the following morning if the later stages of fertilization were required.

To determine the effect of anaesthetics, the rats were anaesthetized with ether, chloroform, ethyl chloride, ethyl alcohol, paraldehyde, "Nembutal," or nitrous oxide. The anaesthesia was controlled at sufficient depth to permit of operative procedures as judged by the disappearance of cutaneous and corneal reflexes. The volatile anaesthetics and nitrous oxide were administered by inhalation, paraldehyde was given orally, and "Nembutal" was injected subcutaneously or intraperitoneally. Nitrous oxide was administered with a carbon dioxide-oxygen mixture, and the "secondary saturation" technique (Hewer 1948) was employed. Adequate doses of "Nembutal" were found to be 3.5-4.0 mg/100 g body weight (subcutaneously) or 3.0 mg/100 g (intraperitoneally) and of paraldehyde to be 0.3 ml/100 g.

Cold-shock treatment was effected by holding a piece of ice against the fallopian tube for about 1 min, with the rat under subcutaneous "Nembutal" anaesthesia. For the application of hot shock, the ovaries and fallopian tubes were brought to the exterior through dorsolateral incisions, and the rat was suspended with its back on the surface of the water in a water-bath. Small clips were attached to the juxta-ovarian fat and these, by their weight, ensured that the fallopian tubes were totally immersed in the water. The temperature of the water was maintained at 44.5-45.5°C and treatment was applied for 8-12 min.

The colchicine solution was freshly prepared each morning and was injected intraperitoneally at the rate of 0.05-0.1 mg/100 g body weight.

Sperm extracts were prepared by suspending rat epididymal sperms in normal saline solution and passing the mixture through a glass bacterial mill.

All eggs were examined in the fresh state; they were dissected from the fallopian tubes under saline, transferred to a slide, slightly compressed under a coverslip with greased edges, and studied with a phase-contrast microscope.

III. Observations

(a) *General Effects of the Selected Anaesthetics*

Among the anaesthetics tried, ether, "Nembutal," and paraldehyde were found to be the most satisfactory for rats, in that surgical anaesthesia of at least 15-30 min duration could be maintained without much risk. On the other hand, chloroform, ethyl chloride, and nitrous oxide were more difficult to use and the resuscitative measures which were frequently needed would have interfered seriously with operative procedures.

Table 1

MEAN RECTAL TEMPERATURE OF FIVE ADULT FEMALE RATS BEFORE, DURING, AND AFTER 15 MIN ETHER ANAESTHESIA

Rectal Temperature (°C)									
Before Anaesthesia	During Anaesthesia			After Anaesthesia					
	5 Min	10 Min	15 Min	5 Min	10 Min	15 Min	30 Min	1 Hr	2 Hr
38·0	34·9	33·0	32·1	31·2	31·5	32·5	35·7	36·9	37·3

Body temperature seemed to fall with all the forms of anaesthesia, but actual measurements were made only when ether or subcutaneous "Nembutal" were used. With ether anaesthesia, body temperature fell progressively and reached a minimum shortly after the end of administration. The temperature then rose gradually until it was approximately normal about 2 hr later. Mean figures for the rectal temperature of five female rats subjected to anaesthesia for 15 min are shown in Table 1. The maximum depression of temperature, observed 5 min after anaesthesia, was about 7°C. With "Nembutal" anaesthesia, the maximum depression of body temperature observed in five female rats was only reached about 2 hr after injection, but was of the same magnitude, nearly 7°C (Table 2). Recovery of body temperature was also slower, and even 5 hr after injection the mean temperature was still 1.5°C below that recorded prior to the injection of the "Nembutal."

Table 2

MEAN RECTAL TEMPERATURE OF FIVE ADULT FEMALE RATS BEFORE AND AFTER SUBCUTANEOUS INJECTION OF "NEMBUTAL"

Rectal Temperature (°C)						
Before Injection	After Injection					
	½ Hr	1 Hr	2 Hr	3 Hr	4 Hr	5 Hr
37·9	34·7	32·0	31·0	33·8	35·3	36·4

Ethyl alcohol was administered in the manner of an anaesthetic, not with this purpose in mind but to achieve a lasting concentration in the body fluids without the risk of lethal intoxication. Rats subjected to alcohol vapour did reach a degree of unconsciousness sufficient for surgical work but only after 1 hr of treatment. The animals were maintained in this state for 2 hr without showing evidence of impending respiratory failure.

(*b*) *The Contraction of the Vitellus*

The volume of the vitellus was determined from measurement of the diameter of uncompressed eggs with an eyepiece micrometer. The mean diameter of 46 eggs from five unmated oestrous rats was 75.5 μ (range 72.8-81.3), equivalent to a volume of 225,000 cu. μ. The effect of sperm penetration upon the volume of the vitellus was observed in five mated rats. The 43 eggs obtained all had sperms within the vitellus. Their mean diameter was 72.1 μ (range 68.5-74.9), equivalent to a volume of 195,000 cu. μ. The difference between the means for diameters is 3.4 μ or about 5 per cent. ($P < 0.01$) and between the volumes 30,000 cu. μ or 13 per cent. Differences of this order were observed also after cold-shock treatment in the eggs from some unmated oestrous rats; the eggs in one fallopian tube were treated while those in the other tube served as controls. Results, however, were not consistent, for, in other rats, both treated and untreated eggs were found to have similar diameters, although second polar body abstriction had occurred in the treated eggs and not in the untreated.

(*c*) *The Induction of Second Polar Body Abstriction*

The artificial induction of abstriction of the second polar body was studied in a total of 120 rats that were treated with anaesthetics and other agents (Table 3). Eggs from 23 untreated rats were also examined, the animals being killed at about the same time after ovulation as those in the test groups; among the 227 eggs that could be analysed, the second maturation division in metaphase was seen in all except two eggs which exhibited polar bodies instead. Three other eggs had polar bodies in addition to the metaphase chromosome group (Plate 1, Fig. 1). There was thus a total of five eggs (2.2 per cent.) with polar bodies; in three eggs (1.3 per cent.) the first polar body had persisted and in two eggs (0.9 per cent.) the second polar body had been formed spontaneously.

A group of 20 rats was treated with ether anaesthesia for 15 min, and provided a total of 186 eggs, of which 183 were analysable (Table 3). Sixty-four per cent. of these eggs had polar bodies; they were clearly second polar bodies, for metaphase chromosomes could not be seen and in most of the eggs the remains of the spindle could still be identified (Plate 1, Fig. 2). The second maturation spindle, in metaphase, was present in the remaining eggs except for one in which the meiosis had not been resumed but the chromosomes had become scattered through part of the cytoplasm. It is possible that chromosome scatter had occurred also in the three eggs that could not be analysed, but it is a difficult process to recognize with certainty.

Some evidence on the rate at which the second polar division occurs was obtained with this group by killing the animals at different times after anaesthesia. Two of the rats were killed 1 hr after the end of anaesthesia. They yielded 17 eggs of which eight exhibited stages in the resumed meiotic division; anaphase was evident in one egg, early telophase in six eggs, and the polar body in one egg. Five other rats were killed at 1½ hr after the end of anaesthesia. These provided 47 eggs, in 32 of which there was a freshly formed polar body; in the remaining 15 the second maturation spindle was still in metaphase. In eggs with recent polar bodies, the group of chromosomes remaining within the vitellus was in the form of a dense rounded mass (Plate 1, Fig. 2). The other 13 rats that received ether anaesthesia were killed 2-3 hr after the end of anaesthesia, and from them were obtained 186 eggs, of which 77 had polar bodies. In most of the eggs with polar bodies, the chromosomes remaining within the eggs were still arranged in a dense mass, but in some a certain amount of scatter had occurred (Plate 1, Fig. 3).

TABLE 3

PROPORTION OF EGGS WITH POLAR BODIES AFTER APPLICATION OF VARIOUS AGENTS *IN VIVO*

Agent	Number of Rats	Total Eggs	Analysable Eggs	Eggs with Chromosome Scatter	Eggs with Polar Bodies	
					Number	Fraction (%)
None	23	230	227	0	5	2·2
Ether: 15 min	20	186	183	1	117	64
"Nembutal" (subcutaneous)	10	89	86	0	1	1·2
"Nembutal" (intraperitoneal)	10	96	89	2	23	26
Chloroform: 15 min	10	95	91	3	40	44
Ethyl chloride: 15 min	10	89	82	1	8	9·8
Ethyl alcohol: 2 hr	10	97	80	15	6	7·5
Paraldehyde (oral)	10	93	83	4	33	40
Nitrous oxide: 15 min	10	93	82	3	45	55
Nitrous oxide: 30 min	10	95	80	0	59	74
Cold shock (ice)	10	91	83	0	82	99
Hot shock (44·5-45·5°C)	10	86	64	10	0	0
Sperm extract	10	77	68	1	7	10

In a second experimental group, rats were given "Nembutal" subcutaneously. Only one of the 86 analysable eggs had a polar body (Table 3). No chromosome scatter was recorded, but it may have occurred in the three eggs that could not be analysed. On the other hand, when "Nembutal" was administered intraperitoneally, 26 per cent. of the analysable eggs had polar bodies and in two eggs with polar bodies chromosome scatter was observed (Table 3).

Similar observations were made on the effects of anaesthesia with chloroform (15 min), ethyl chloride (15 min), ethyl alcohol (2 hr), paraldehyde, and nitrous oxide (15 and 30 min); 10 rats were used with each agent (Table 3). The proportion of analysable eggs that contained polar bodies varied greatly, from 7.5 per cent. with ethyl alcohol to 74 per cent. with 30-min nitrous oxide. With each of these anaesthetics, there were some eggs that lacked polar bodies but in which chromosome scatter could be distinguished. Particularly was this so with alcohol; rats treated with this agent gave 15 eggs that showed chromosome scatter but no spindle or polar body.

The most effective method for inducing the emission of the second polar body was found to be cold-shock treatment. Ten rats so treated gave 83 analysable eggs, of which all but one had polar bodies (Table 3); the single exception was a degenerate egg.

TABLE 4

CORRELATION BETWEEN MALE AND FEMALE ELEMENTS DURING THE EARLY STAGES OF FERTILIZATION IN 387 EGGS FROM ADULT RATS

Female Male	Chromosomes in Metaphase	Chromosomes in Anaphase	Chromosomes in Telophase	Polar Body Formed	Early Female Pronucleus Formed	Total
Sperm in perivitelline space	93					93
Sperm in vitellus. Head unchanged	24	13	4			41
Sperm in vitellus. Head changing	5	14	51	14		84
Sperm in vitellus. Head invisible			8	70	1	79
Early male pronucleus formed				9	81	90
Total	122	27	63	93	82	387

Rats treated with anaesthetics and with cold shock were killed at intervals between 1 and 4 hr thereafter, and in this way it has been possible to examine eggs at various times after the induction of polar body abstriction. Observations at 1 and 1½ hr were those made after ether anaesthesia and have already been described. With the other agents also, polar body formation was complete in less than 2 hr and the chromosomes remaining in the vitellus were generally compacted into a dense mass (Plate 1, Fig. 2). In some eggs, chromosome scatter, following polar body abstriction and resembling that shown in Plate 1, Figure 3, was observed. When eggs were examined later, 3-4 hr after treatment, increasingly more of them showed chromosome scatter, and in progressively more eggs with polar bodies no chromosomes could be distinguished, probably because they were scattered through portion of the vitellus.

Rats that received the hot-shock treatment provided 64 eggs that could be analysed and none of these showed a polar body. In 10 eggs neither spindle nor polar body could be seen but scattered chromosomes were discernible in the cytoplasm.

Sperm extracts were injected into the peri-ovarian sac of 10 rats. Among the 68 analysable eggs recovered, seven had polar bodies and one egg showed chromosome scatter.

(*d*) *Correlation of Male and Female Elements in the Early Stages of Fertilization*

Information on the correlation between the changes undergone by the sperm head and its derivatives on the one hand and the egg chromosomes and their derivatives on the other during fertilization has already been reported (Austin 1951). The observations were made on eggs recovered from immature rats after induced ovulation. Data have now been obtained on the eggs of adult rats. One hundred and forty-four adult female rats were mated between 9.00 and 9.30 a.m. and killed 2½-4 hr and 7 hr after mating. The successive changes shown by the male and female elements were classified in a similar manner to that used by Austin (1951), except that an extra stage was introduced: that in which the sperm head, having undergone some degree of metamorphosis, is temporarily indistinguishable by phase-contrast microscopy as it lies in the cytoplasm.

The data are set forth in Tables 4, 6, and 7. Sixty-seven rats provided a total of 387 eggs in the early stages of fertilization. There were 93 eggs in which sperms were present only in the perivitelline space, and in all these eggs the second maturation spindle was still in metaphase. In 41 eggs, sperms had entered the vitellus but had not undergone any detectable change; many of these eggs (24) displayed metaphase chromosomes but in others the meiosis had advanced to anaphase (13 eggs) or to telophase (four eggs). The early changes that the sperm head undergoes in the vitellus were seen in 84 eggs, the majority (51) of which had the second maturation spindle in telophase. In some (14) of the eggs, however, polar body abstriction had been completed, whereas in others there was evidence of delay in the response of the egg to sperm penetration, for the meiosis was still in anaphase (14 eggs) or even in metaphase (five eggs). The stage in which the sperm head is indistinguishable was noted in 79 eggs and in most (70) of these the polar body had been formed but the group of chromosomes remaining within the egg had not yet given place to pronuclei. The female pronucleus was evident in only one egg in this stage, while in eight others the chromosomes were still arranged in telophase. Early male pronuclei were recorded in 90 eggs altogether and in 81 of these the female pronucleus was also discernible. The remaining nine eggs had a polar body but no female pronucleus.

(*e*) *Induction of Polar Body Abstriction before Fertilization*

The capability for fertilization of eggs in which polar body formation had been artificially induced was tested by the use of ether, cold shock, and nitrous oxide. The results are shown in Table 5.

A control group of 36 rats, mated under the same conditions as the animals in the test groups, yielded a total of 327 eggs, of which 294 had been penetrated by sperms, 115 of them by more than one sperm. All the penetrated eggs were undergoing apparently normal fertilization or early cleavage, except for 27 eggs (9.2 per cent.) that were polyspermic but otherwise normal (25 dispermic, two trispermic). There were 88 eggs in the pronuclear stages of fertilization.

TABLE 5

EFFECT ON FERTILIZATION OF ANAESTHETICS AND OF COLD-SHOCK TREATMENT WHEN APPLIED TO MATED RATS BEFORE SPERM PENETRATION

Agent	Number of Rats	Total Eggs	Penetrated Eggs		Eggs with Pronuclei			Eggs in Other Stages of Fertilization or in Cleavage
			Number	Fraction (%)	Total	Normal ♂ Pronuclei	Normal ♀ Pronuclei	
None	36	327	294	90	88	88	88	206
Ether: 15 min	21	196	91	46	30	29	24	61
"Nembutal" (subcutaneous)	10	89	71	80	17	16	16	54
Cold shock	15	130	52	40	25	23	17	27
Nitrous oxide: 30 min	6	58	47	81	23	23	17	24
Colchicine	16	129	97	75	43	40	32	54
Hot shock	17	168	137	82	55	52	41	82

Ether anaesthesia of 15 min duration was administered to rats after mating and these provided 91 penetrated eggs. In most of the eggs in the pronuclear stages of fertilization both the male and female pronuclei were adjudged to be normal. Some, however, showed pronuclear abnormalities; in one egg both pronuclei were degenerate and in another five, one pronucleus, considered to be the male pronucleus, was normal whereas the female pronucleus was either absent altogether or replaced by one to three subnuclei. The appearance of subnuclei is shown in Plate 1, Figure 5, and Plate 2, Figures 8 and 9; they are smaller than pronuclei and may contain only one nucleolus. The rats in this group also provided eggs that either showed stages in the formation of the first cleavage spindle or else had undergone cleavage and were in the 2-cell stage; all appeared quite normal.

A third group of rats was treated similarly to the second group, except that they received a subcutaneous injection of "Nembutal" in place of ether anaesthesia. This group provided 71 penetrated eggs that were in various stages of fertilization or early cleavage, and only one egg showed any abnormality. It was polyspermic, for there were two sperm mid-pieces in the vitellus, but it had only a single large oval nucleus and five subnuclei (Plate 1, Fig. 4). The oval nucleus may possibly have been formed by the fusion of two male pronuclei.

In a fourth experimental group the eggs were subjected to cold-shock treatment *in vivo*, between the times of mating and anticipated sperm penetration. Here again, distinct abnormality could only be seen in some of those in the pronuclear stages of fertilization. There were 17 eggs in which both male and female pronuclei were considered to be normal, and six in which only the male pronucleus was normal, the female pronucleus being replaced by one to three subnuclei. One exceptional egg had 13 subnuclei of various sizes and another had no distinct nuclei but simply a number of nucleoli scattered through the cytoplasm. The rats in this group also provided eggs that showed the first cleavage spindle or had entered the 2-cell stage; all were quite normal in appearance.

TABLE 6

PROPORTION OF EGGS WITH POLAR BODIES AT TWO DIFFERENT STAGES OF FERTILIZATION IN UNTREATED RATS AND AFTER TREATMENT WITH COLCHICINE OR HOT SHOCK

Treatment	Eggs with Sperm Head Invisible			Eggs in Pronuclear Stage			
	Total	With Polar Body		Total	Pronuclei Normal		
		Number	Fraction (%)		Total	With Polar Body	
						Number	Fraction (%)
None	79	71	90	88	88	88	100
Colchicine	34	4	12	43	32	3	9
Hot shock	28	8	29	55	41	37	90

In the fifth group, rats anaesthetized for 30 min with nitrous oxide yielded 40 penetrated eggs in which fertilization appeared to be proceeding normally. Two other penetrated eggs, however, contained only one pronucleus (considered to be the male), and four had an apparently normal male pronucleus but one or more subnuclei in place of the female pronucleus. An egg with three visible subnuclei is illustrated in Plate 1, Figure 5.

Among the 190 penetrated eggs from the rats that received ether or nitrous oxide anaesthesia or cold-shock treatment there were 19 polyspermic eggs, of which 15 were dispermic and four trispermic. This incidence does not differ significantly from the 9.2 per cent. observed in the control group of rats.

(*f*) *Inhibition of the Second Maturation Division*

An attempt was made to prevent the occurrence of the second maturation division, which leads to the abstriction of the second polar body and which normally follows sperm penetration. This was done by giving the rats an intraperitoneal injection of colchicine shortly after mating, or by subjecting the eggs to hot-shock treatment *in vivo,* between the times of mating and fertilization. The rats were killed in the late afternoon or on the following morning. The results are set forth in Tables 5, 6, and 7.

TABLE 7

NUMBERS OF EGGS PENETRATED BY MORE THAN ONE SPERM IN UNTREATED RATS AND AFTER COLCHICINE AND HOT-SHOCK TREATMENTS AND THE PROPORTIONS OF THOSE EGGS THAT WERE POLYSPERMIC

Treatment	Total Penetrated Eggs	Eggs Penetrated by >1 Sperm		Polyspermic Eggs		B/A (%)
		Number, A	Fraction (%)	Number, B	Fraction (%)	
None	294	115	39	27	9·2	23
Colchicine	97	28	29	9	9	32
Hot shock	137	34	25	22	16	65

The rats that received colchicine yielded 97 penetrated eggs, of which nine were polyspermic. The polyspermic eggs represented 32 per cent. of eggs that contained more than one sperm. Among the eggs undergoing fertilization, there were 34 eggs wherein the head of the sperm had reached the stage at which it is indistinguishable in the cytoplasm, but in the majority of these no polar body had been formed. Clearly, polar-body abstriction had been inhibited by the colchicine, and this effect was evident also in 32 apparently normal pronucleate eggs, none of which had polar bodies. Abnormal eggs included three in which only subnuclei or groups of nucleoli were present, five that showed a single large, presumably male, pronucleus (Plate 2, Fig. 7), and three eggs that had one or two subnuclei in place of the female pronucleus (Plate 2, Fig. 9).

From the rats treated by the hot-shock method, 137 penetrated eggs were recovered. Thirty-four of the penetrated eggs had more than one sperm within the bounds of the zona pellucida, and 22 (65 per cent.) of these had more than one sperm within the vitellus, that is, they were polyspermic (19 eggs were dispermic and three trispermic). Of the eggs that showed the stage of fertilization at which the sperm head is invisible, the majority (71 per cent.) had no polar bodies, but of the eggs that were recovered later, in normal pronuclear stages, nearly all (90 per cent.) possessed polar bodies. The inhibitory effect of hot shock, unlike that of colchicine, appears to pass off in a few hours. Fourteen normally cleft 2-cell eggs were obtained from rats in this group; 10 of the eggs had polar bodies. The abnormal eggs included seven with only one large nucleus (like that in Plate 2, Fig. 7), presumed to be the male pronucleus, and seven with apparently normal male pronuclei with one or more subnuclei in place of the female pronucleus.

IV. Discussion

In confirmation of the findings of Sobotta and Burckhard (1910), Kirkham and Burr (1913), and Huber (1915), the first polar body was rarely seen in the recently ovulated eggs of untreated rats. The incidence observed in 227 eggs was 1.3 per cent. Sometimes the second maturation meiosis is resumed spontaneously, for in 0.9 per cent. of the eggs the second polar body was evident. The two types of polar body have the same significance for the interpretation of many of the observations described in this paper, since it was not possible to distinguish between them in eggs that showed chromosome scatter or were in the later stages of fertilization. Moreover, the incidence of polar bodies in the eggs from the untreated rats was low, and any significant increase in the incidence could therefore be attributed to the emission of the second polar body resulting from the treatment applied.

Results also confirm Thibault's (1949) observation that ether anaesthesia causes the emission of the second polar body. He found polar bodies in 76 per cent. of the eggs after 15-20 min anaesthesia; in the present investigation 64 per cent. of the eggs had polar bodies after 15 min anaesthesia. Thibault ascribed the effect partly to a direct action of ether upon the eggs and partly to the fall in body temperature that occurs during the anaesthesia. The authors' observations favour a direct action upon the eggs, or upon the immediate environment of the eggs, for the fall in body temperature was equally profound and even more protracted when narcosis was induced with subcutaneously injected "Nembutal" and yet the proportion of eggs with polar bodies was not increased above that seen in untreated rats. When the "Nembutal" was given by the intraperitoneal route, however, a moderately high proportion (26 per cent.) of the eggs was found to have polar bodies. By this method of administration, the "Nembutal" may well achieve a higher concentration in the immediate environment of the eggs and a direct action upon this environment or even upon the eggs themselves becomes more likely. The precise nature of the action is uncertain but may involve local anoxia. A high proportion of eggs with polar bodies was observed after nitrous oxide anaesthesia, and with this agent the predominating, perhaps exclusive, effect at the cellular level is the production of anoxia, particularly when the secondary saturation technique is employed. Various proportions of eggs were found with polar bodies after treatment with other anaesthetics, corresponding perhaps to differing degrees of cellular anoxia produced.

Both hot and cold stimuli have been found to induce activation, including the emission of the second polar body, in the eggs of some invertebrates and lower vertebrates (Tyler 1941; Peacock 1943), and in the rabbit (Pincus and Enzmann 1936; Pincus 1939). The two forms of temperature shock had very different effects on rat eggs, for whereas cold shock induced polar body formation in virtually all the eggs, as Thibault (1949) also reported, no eggs were found with polar bodies after the hot-shock treatment. Indeed, it was shown that hot shock will inhibit, for a while, the second polar meiosis that normally follows sperm penetration.

In some of the eggs from rats treated with anaesthetics and other agents, there was no sign of a resumption of the second maturation meiosis, but instead the chromosomes were found to have become scattered through the neighbouring cytoplasm. This was seen particularly after treatment with ethyl alcohol and hot shock. Chromosome scatter is not a normal consequence of sperm penetration and has not been observed to occur spontaneously during the 24 hr after ovulation. It is evidently an aberrant reaction on the part of the egg to the stimulus applied.

A close correlation between the changes undergone by the male and female elements during the early stages of fertilization was found in untreated adult rats, and in general the results were similar to those previously reported for immature rats (Austin 1951). The chief difference was that the eggs of adult rats appear less sensitive to the stimulus of sperm entry than do those of immature rats, for in no instance did the resumption of meiosis occur before the sperm had penetrated the vitellus. Indeed in 29 eggs the second maturation spindle was still in metaphase although the sperm head lay in the vitelline cytoplasm and in five of these eggs the sperm head had even begun its early metamorphosis.

Confirming earlier observations (Austin 1952), sperm penetration was noted in a large proportion (90 per cent.) of the eggs from rats that were not permitted to mate until several (5-9) hours after ovulation. The incidence of polyspermy in these eggs was 9.2 per cent., a figure identical with that recently reported for another group of rats mated after ovulation (Austin and Braden 1953*a*). This high incidence of polyspermy is associated with delayed mating; after normal mating the figure was only about 1.5 per cent. (loc. cit.).

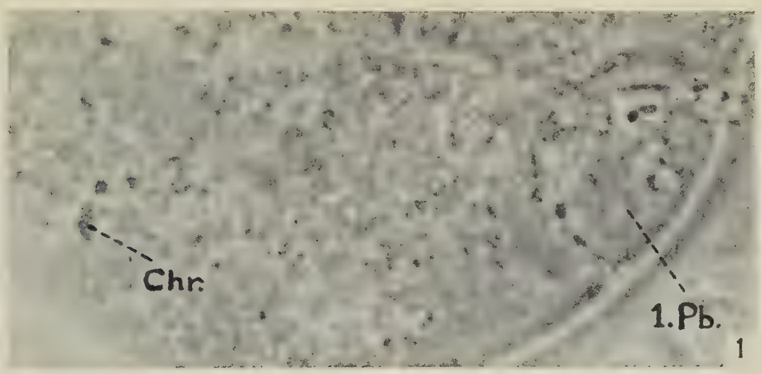
Procedures designed to induce second polar body abstriction (ether or nitrous oxide anaesthesia, or cold shock) were applied to mated rats before the time of sperm penetration and resulted in a reduction in the number of eggs penetrated. Nevertheless, it was quite clear from the results that sperms could penetrate into eggs that had already formed the second polar body. Moreover, the mechanism underlying the block to polyspermy was evidently little affected, for the incidence of polyspermy in these rats (10 per cent.) differed insignificantly from that observed in the control group. Apparently normal fertilization may also occur, in spite of the initial disturbance in the correlation between the male and female elements. It would seem that this disturbance was corrected before the formation of the female pronucleus, but only if chromosome scatter had not taken place. The development of subnuclei in place of normal female pronuclei was probably the result of chromosome scatter. Of interest is the fact that the rabbit egg also, according to Chang (1952), may be fertilized and show early cleavage after chilling *in vitro*, but Chang could not be certain that the emission of the second polar body had preceded sperm penetration.

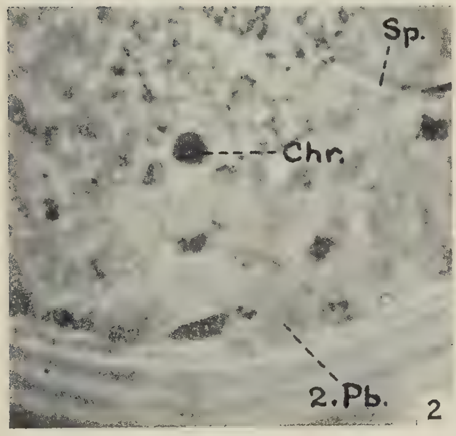
Emission of the second polar body normally follows sperm penetration but could be inhibited with either hot shock or colchicine. The results differed according to the method employed. The inhibitory influence of hot shock apparently passed off in most of the eggs, so that polar body abstriction, fertilization, and the first cleavage could then proceed in a normal manner. Once again the initial disturbance in the correlation between male and female elements seems to have been adjusted before pronuclei were formed. Sometimes subnuclei replaced the female pronucleus, presumably owing to chromosome scatter. The influence of colchicine, on the other hand, was more lasting, and, after treatment with this agent, the majority (90 per cent.) of eggs with seemingly normal pronuclei showed no sign of having emitted the second polar body. It may well be that such eggs contained a diploid female pronucleus and were thus capable of giving rise to a triploid embryo, but further work is required to explore these possibilities.

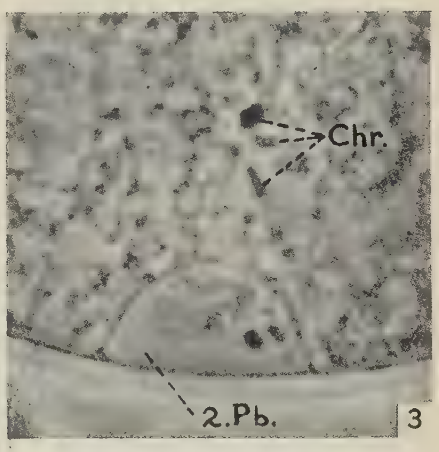
The effect of hot shock on rat eggs was unlike that observed by Fankhauser and Godwin (1948) in *Triturus*; in this species, the treatment caused submergence of the spindle and inhibited the formation of the second polar body but did not prevent the resumption of the meiosis following sperm penetration. Two female pronuclei were thus formed and both subsequently underwent syngamy with the male pronucleus. Fankhauser and Godwin showed that it was by this mechanism that hot shock led to the production of triploid embryos. Fischberg and Beatty (1952) found a large increase in the incidence of triploidy following hot-shock treatment in mice and thought the mechanism was probably the same as in *Triturus*. Results obtained in the present investigation do not support this hypothesis, at least for the rat egg. On the other hand, a striking effect of hot shock upon the rat egg was found to be an increase in the incidence of polyspermy; among the eggs so treated, 16 per cent. were polyspermic compared with 9.2 per cent. in the control group. Examination of the data shows that 65 per cent. of the eggs that contained more than one sperm were polyspermic, as compared with 23 per cent. in the control group. These facts are interpreted as showing that hot shock interferes with the operation of the block to polyspermy, and there is evidence that dispermy would lead to triploidy in the embryo (Austin and Braden 1953*b*). By contrast, colchicine had little if any influence on this response of the eggs to sperm penetration. It would seem that both colchicine and hot-shock treatment may give rise to polyploid rat embryos, the first by suppression of the second polar division and the second by rendering the egg more susceptible to polyspermy.

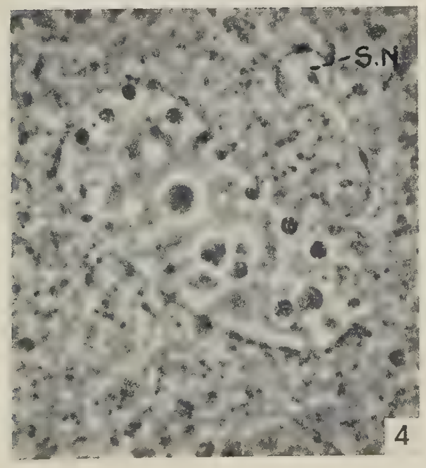
A small proportion of the eggs obtained from mated rats that had received treatment with anaesthetics, temperature shock, or colchicine were classed as abnormal. The fate of these eggs is worthy of speculation, for they were seldom actually degenerate in appearance and some may well have been capable of further development. Of particular interest were 15 eggs in which only a single large nucleus, considered to be the male pronucleus, was present (as in Plate 2, Fig. 7), and seven eggs that had a male pronucleus and two subnuclei in place of the female pronucleus (as in Plate 2, Fig. 8). Rat eggs having only the male pronucleus may be able to undergo subsequent cleavage, just as, apparently,

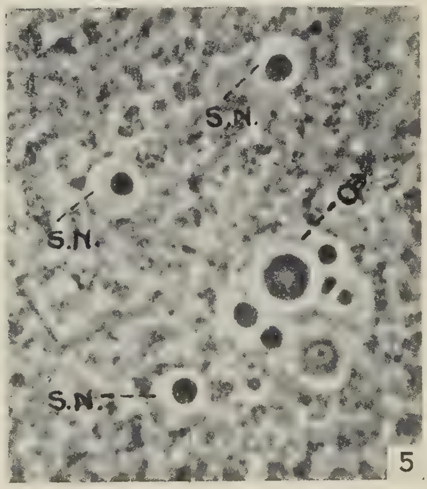
SECOND POLAR DIVISION IN THE RAT EGG











rabbit eggs can with only a female pronucleus (Thibault 1949), but whereas the rabbit eggs are diploid, the rat eggs are presumably haploid and could thus give rise to haploid embryos. Some of the eggs with two well-formed subnuclei in addition to the male pronucleus showed no evidence of having abstricted the second polar body. They could therefore have arisen in the manner described by Fankhauser and Godwin (1948) for *Triturus* and would theoretically be capable of giving rise to triploid embryos.

Observations show that in the rat the immediate consequences of sperm penetration, which may be referred to as the criteria of activation, are independent processes and are capable of separate evocation. Both the contraction of the vitellus and the emission of the second polar body can be induced by non-specific stimuli. Moreover, the resumption of the second maturation division may also be inhibited by artificial means. These things may be done, however, without seriously interfering with the penetrability of the vitellus to the sperm or with the operation of the block to polyspermy. As yet it has not been found possible to induce the block to polyspermy by artificial means. Only the entry of the sperm was observed to evoke this reaction in the rat egg, which thus differs from the eggs of certain lower animals.

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Explanation of Plates 1 and 2

All the photographs were obtained with the aid of a phase-contrast microscope. Magnification is × 1250 for Figures 1, 2, and 3, and × 1000 for Figures 4-9.

Plate 1

Fig. 1.—A persisting first polar body (1. *Pb.*) and the metaphase chromosome group (*Chr.*) of the second maturation division.

Fig. 2.—A newly formed second polar body (2. *Pb.*) in an egg undergoing fertilization. The dense rounded mass of chromosomes (*Chr.*) in the vitellus and the equatorial plate of the spindle are clearly visible. (*Sp.* = sperm mid-piece.)

Fig. 3.—A second polar body about 4 hr after its formation was artificially induced. The chromosomes (*Chr.*) in the vitellus are somewhat scattered and there is no trace of the spindle.

Fig. 4.—A polyspermic egg obtained from a rat subjected to narcosis by subcutaneous injection of "Nembutal." The single large oval nucleus may be the product of fusion of two male pronuclei. Apart from this, only subnuclei were present, one of which is visible in the figure.

Fig. 5.—Egg from a rat treated with nitrous oxide anaesthesia, showing the male pronucleus and three of the subnuclei (*S.N.*) that apparently replace the female pronucleus.

Plate 2

Fig. 6.—The male and female pronuclei in a normal egg undergoing fertilization; they are shown here for comparison with the pronuclei and subnuclei in Figures 7-9.

Fig. 7.—The sole nucleus to be seen in a penetrated egg from a rat treated with hot shock after mating. It is presumed to be the male pronucleus. (*Sp.* = sperm mid-piece.)

Figs. 8 and 9.—The apparently normal male pronucleus and the two large subnuclei (*S.N.*) that replaced the female pronucleus in eggs obtained from rats after treatment with cold shock (Fig. 8) and colchicine (Fig. 9).

THE PHYSIOLOGY OF GROWTH IN APPLE FRUITS

VII. BETWEEN-TREE VARIATION OF CELL PHYSIOLOGY IN RELATION TO DISORDER INCIDENCE

By D. Martin,* T. L. Lewis,* and J. Cerny*

[*Manuscript received March* 25, 1954]

Summary

Mean cortical cell size, soluble and protein nitrogen per cell, preclimacteric respiration, mean fruit size, and incidence of disorders have been studied for fruit of each tree in a plot of 35 trees of Jonathan variety. These trees were remarkably uniform with regard to soil, aspect, tree size, and pollinating variety, but provided a range of mean fruit size per tree.

There was a high degree of correlation between the variables. Cortical cell size increased with mean fruit size but more rapidly than would be expected from a proportional increase with size of fruit. Protein nitrogen increased proportionally with cell volume but the ratio of protein nitrogen and cell surface increased with cell size, suggesting that the protoplasm increased in thickness with cell size. Intercorrelation between respiration per cell, protein nitrogen, soluble nitrogen, and cell size were particularly close, remaining highly significant even when mean fruit size per tree was held constant by methods of partial correlation analysis, suggesting that these characteristics are functions of cell growth and are not influenced by between-tree differences due to cropping. Disorder incidence is correlated with the other variables and the implications of these relationships are discussed.

I. Introduction

The keeping quality of fruit is determined while it is on the tree. Study of the between-tree variation in fruit physiology under uniform cultural conditions may thus be a useful approach to the improvement of keeping quality.

Earlier work (Martin 1954) showed that the best index of susceptibility to disorder in fruits from different trees was the mean fruit size per tree. This was better than any of the other indices associated with ripening (e.g. acid and soluble solids concentration, starch or ground colour change) and it was not improved when combined with any of these indices. Martin and Lewis (1952) showed that between varieties, cell volume, respiration per cell, protein nitrogen per cell, and respiration rate per unit protein (Hulme 1951) were positively correlated.

Robertson and Turner (1951) showed that, in fruit maturing on a single tree, protein synthesis kept pace with cell enlargement and they put forward the hypothesis that higher protein contents made greater demands on the energy distributors of the cells and resulted in higher respiration rates.

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These findings suggested that a study of one variety over a range of crop sizes might help in the elucidation of the remarkably close relation between mean fruit size per tree and breakdown incidence, and might be a useful introduction to attempts to improve keeping quality.

II. Material and Methods

In the 1951-52 season a block of Jonathan trees, 30 yr old and exceptionally uniform with regard to tree size, soil type, and slope became available. It consisted of six adjacent rows of trees planted 16 by 16 ft extending across the orchard with Cleopatra variety for the next six rows adjacent. The mean crop per tree over 4 yr ranged from 600 to 1000 fruits. The trees received no manuring for the season 1950-51 and the 35 trees used for this study were not manured for the 1951-52 season.

On March 19, 1952 a sample of 20 fruits and another of 150-200 fruits were taken by a procedure designed to produce random sampling. The former was used to determine respiration rate at 25°C by a modification of the method of Eaves (1938), taking the mean rate for the 40-48 hr after picking as did Hulme (1951). This respiration was preclimacteric. From 10 of these fruits a transverse section was cut from the mid-cortex region of each fruit midway between stem and calyx, fixed in formalin-acetic-alcohol, stained with ruthenium red, and mounted in "Euparal." Mean cell size was determined on the basis of the work of Bain and Robertson (1951) and by a sampling method of cell ranking devised by McIntyre (1953). This method was about twice as efficient as random sampling for the same number of cells measured. The mid-cortical tissue of the 20 fruits was sliced and dried at 65°F, powdered, and stored in sealed jars at 32°F for protein analysis by the methods used previously (Martin and Lewis 1952).

The larger samples of 150-200 fruits were stored at 33-34°F for 7 months, when they were removed to room temperature. Mean fruit size was then determined and the fruits were examined for disorders immediately, and again after 2 wk at room temperature.

The use of samples of differing sizes introduces difficulties in the mathematical treatment which will be referred to later. Mean fruit size and disorder incidence are easily determined and on a sample of 150-200 fruits involve sampling standard errors relative to the mean of only ± 1 per cent. in mean fruit size and ± 3 per cent. in disorder.

Respiration rate and protein and soluble nitrogen contents are determined on samples of only 20 fruits; from a limited number of duplicates it is estimated that the coefficient of variation for bulked material from 20 fruits is of the order of 5·5 per cent.

Determinations of mean cell size from two samples of 10 fruits gave results which differed by 1 per cent. The sampling error in the determination of mean cortical cell size is of the order of ± 5 per cent. Mean cell size can be regarded in two ways: (*a*) as an estimate of mean cell size of the cortical tissue; and (*b*) as an estimate of mean cell size for the whole fruit. This estimate of mean cell size for the whole fruit is based on the work of Bain and Robertson (1951) and the validity of a comparison between trees requires the assumption that the cell size gradient within fruits did not differ between trees. As the range of mean fruit size between trees in this plot was not great (83-113 g), we consider this assumption justified, and a comparison of mean cell number per fruit to be possible by their methods.

Respiration rate cannot be determined from the cortical tissues only. However, the rate for the whole fruit cannot be very different from that for the cortical tissues only as the cortex is approximately 80 per cent. of the total respiring tissue.

III. Results

The data for the 35 trees are set out in Table 1, and correlation coefficients for a number of variables are given in Table 2. Some of the correlations are examined further by methods of partial correlation in Table 3.

(*a*) *Disorder Incidence and Fruit Attributes*

(i) *Mean Fruit Size.*—The correlation of mean fruit size and percentage breakdown provides further evidence for the accuracy of mean fruit size per tree as an index of breakdown susceptibility in the fruit.

(ii) *Cell Size.*—The correlation of cell size and disorder incidence supports the suggestion in an earlier paper in this series (Martin and Lewis 1952) that variations in disorder level might be related to difficulties of cell organization associated with cell growth.

(iii) *Respiration per Unit Protein.*—This attribute was determined prior to storage, and disorder incidence was the result of 7 months storage, during which respiration proceeded at some function of the prestorage rate. The correlation of these two variables, which are separated by such a long time interval, heightens the possibility that disorder incidence might be determined by difficulties in protein maintenance; the differences in respiration rate per unit protein over the storage period determining the relative depletion of reserves and the extent of breakdown.

(*b*) *Relation of Rots Developing in Store to Mean Fruit Size*

Positive correlation occurred between percentage rots visible when the fruit was removed from store and mean fruit size; with further infections during the period at room temperature, the correlation declined to insignificance. The relationship has not been noted before and while not relevant to the main theme, is reported here as a factor which should be considered in all experiments on rotting where different populations of fruit are compared.

(*c*) *Interrelation of Other Fruit Attributes*

(i) *Mean Fruit Size, Cell Size, and Cell Number per Fruit.*—There was a high positive correlation between mean fruit size and mean cell size but there

TABLE 1

FRUIT ATTRIBUTES OF PLOT OF 35 JONATHAN TREES

Mean Fresh Weight (g)	Disorder (%) = 100 − Sound	Breakdown (%)	Breakdown $2\frac{1}{2}$ in. Fruit (%)	Rots in Store (%)	Jonathan Spot (%)	Cell Volume (cu. mm. × 10^{-5})	Cell Surface (sq. cm. × 10^{-4})	Cell Number per Gram	Respiration per Cell (mg × 10^{-11} CO_2/hr)	Protein Nitrogen per Cell (g × 10^{-14})	Respiration/Unit Protein (mg × 10^{-3} CO_2/g protein N/hr)	Soluble N/Cell (g × 10^{-14})
1	2	3	4	5	6	7	8	9	10	11	12	13
83·7	14·9	10·9	14·0	0·9	17·2	180	9·67	505	463	415	112	177
88·3	34·3	23·7	23·0	4·0	15·2	176	9·60	515	433	389	120	125
88·1	20·3	2·7	3·0	2·2	18·5	148	8·77	622	448	335	134	96
88·0	32·2	13·2	12·0	4·0	19·5	130	8·15	700	380	320	121	94
90·8	45·4	27·6	49·0	5·4	24·9	151	8·94	602	510	357	143	145
91·5	39·5	15·7	29·0	2·3	36·5	183	9·89	496	523	419	125	155
92·0	42·5	26·4	36·0	1·1	25·3	171	9·46	532	500	383	130	98
93·4	39·9	25·8	32·0	1·2	21·5	155	8·99	586	538	365	148	124
93·4	46·8	20·7	34·0	1·8	36·7	171	9·45	582	532	363	147	113
95·0	60·5	59·7	64·0	1·8	14·6	130	8·20	700	381	300	127	112
95·2	37·5	14·7	19·0	7·6	25·9	181	9·73	503	548	403	137	214
95·2	43·0	32·3	43·0	2·5	19·0	164	9·31	553	374	374	118	90
97·5	48·4	38·5	50·0	3·7	14·8	154	8·99	590	485	394	123	128
97·7	53·8	2·8	31·0	3·9	44·9	200	10·27	455	830	475	171	195
97·7	51·5	34·6	41·0	5·0	25·8	180	9·71	505	659	441	150	163
98·0	56·5	39·5	47·0	6·2	18·1	186	9·77	489	694	442	157	228
98·7	58·6	39·4	56·0	2·6	27·7	179	9·68	507	612	392	156	140
100·6	49·0	35·6	41·0	5·0	15·9	187	9·98	485	627	440	143	148
100·7	55·8	32·8	35·0	3·4	44·8	220	10·90	413	852	498	171	309
101·3	62·3	46·3	58·0	3·2	27·8	221	11·07	412	771	520	148	260
101·2	58·1	42·7	48·0	8·0	13·2	216	10·69	420	746	494	151	181
101·9	60·2	24·7	32·0	4·2	46·4	168	9·40	540	501	355	142	165
102·5	74·4	49·7	56·0	4·8	44·9	203	10·44	448	772	460	168	146
102·9	57·4	26·9	29·0	2·4	38·9	174	9·46	522	597	417	143	142
103·5	73·1	44·9	51·0	7·8	41·6	174	9·50	522	672	452	147	191
103·5	51·8	36·0	45·0	10·8	7·9	225	10·44	403	833	552	151	298
105·0	51·9	35·0	43·0	7·3	21·2	196	10·10	463	666	490	136	235
104·3	56·0	40·5	40·0	10·0	13·5	202	10·20	450	765	483	150	195
104·5	68·0	54·6	54·0	5·3	17·3	215	10·85	422	690	485	143	153
105·8	63·6	53·7	58·0	4·6	13·0	231	11·23	394	882	939	164	305
107·7	89·0	78·6	83·0	6·2	29·0	263	12·13	396	980	600	160	291
108·4	75·3	46·6	59·0	6·0	32·7	224	10·93	406	855	482	172	216
109·7	90·1	68·3	71·0	5·0	46·7	256	11·81	355	1085	605	172	287
112·7	83·8	82·3	82·0	6·8	6·2	250	11·60	364	959	531	181	262
112·7	80·0	69·8	66·0	6·8	16·7	217	10·51	418	900	551	163	240

was a significant increase in the residual variation if the fitted regression line of cell size on fruit size was made to pass through the origin. The mean cell size in the cortical tissues increased more rapidly than would have been expected from a proportional increase with the size of the fruit. If the mean cortical cell size is taken as an estimate of the mean cell size of the whole fruit, the converse aspect of this relation is the negative correlation of the total cell number with fruit size (see Table 2). This implies the possibility that the stimulus to cell division may be weaker in light-crop fruitlets; light-crop trees may have not only fewer fruit buds but less cell division in the fruits although they are larger. This point is being investigated further.

TABLE 2

CORRELATIONS OF VARIABLES

Correlation	r	Significance
Mean fruit size and percentage disorder	0·9034	$P<0.001$
Mean fruit size and percentage breakdown (all fruit)	0·7928	$P<0.001$
Mean fruit size and percentage breakdown (2½ in. fruit)	0·6505	$P<0.001$
Mean fruit size and percentage Jonathan spot . .	0·1003	N.S.
Mean fruit size and percentage rots developed during cool storage	0·6157	$P<0.001$
Mean fruit size and percentage rots at final examination	0·2505	N.S.
Mean fruit size and mean cell volume	0·7613	$P<0.001$
Mean fruit size and mean cell surface	0·7373	$P<0.001$
Mean fruit size and cell number per fruit	−0·4452	$P<0.01$
Mean fruit size and respiration per cell	0·8320	$P<0.001$
Mean fruit size and protein N per cell	0·7822	$P<0.001$
Mean fruit size and soluble N per cell	0·6677	$P<0.001$
Mean fruit size and respiration/protein	0·7480	$P<0.001$
Mean cell surface and protein N per cell	0·9328	$P<0.001$
Protein N per cell and respiration per cell . .	0·9371	$P<0.001$
Protein N per cell and R/P ratio	0·6705	$P<0.001$
Protein N per cell and soluble N per cell	0·8661	$P<0.001$
Protein N per cell and percentage breakdown . .	0·7010	$P<0.001$
Percentage disorder and R/P ratio	0·7513	$P<0.001$
Percentage breakdown and mean cell volume . .	0·6416	$P<0.001$
Percentage breakdown and R/P ratio	0·5336	$P<0.001$
Percentage breakdown and percentage Jonathan spot	0·5020	$P<0.001$

(ii) *Mean Cell Volume, Cell Surface, and Protein Nitrogen.*—Over the range of mean cell sizes provided by these data the relation of cell volume to cell surface was linear (Fig. 1) owing to the tendency for the cells to increase faster along the major axis than along the minor axis.

The relation of protein nitrogen per cell to cell volume was particularly close, remaining highly significant when mean fruit size per tree was held constant. The protein nitrogen/cell volume line (Fig. 2) passed through the origin or very close to it, showing a proportionality between protein and cell volume.

The ratio of protein nitrogen to cell surface increased as cell surface increased (Fig. 3), suggesting either that the protoplasm of the cortical cells increased in thickness as cell size increased or that the protoplasm became more concentrated.

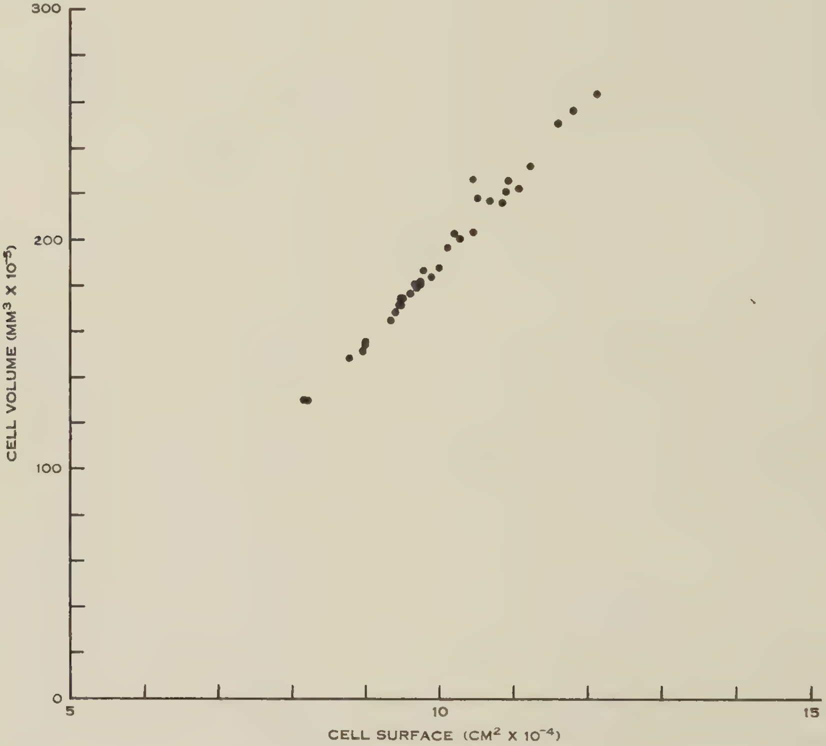

Fig. 1.—Cell surface and cell volume.

(iii) *Protein Nitrogen, Soluble Nitrogen, and Respiration.*—The close relation between protein nitrogen per cell and preclimacteric respiration per cell illustrates the interdependence of these two factors over a range of crop levels. Because the relation between cell volume and cell surface was linear over the range available (Fig. 1), soluble nitrogen was linearly related to both cell volume and cell surface (Figs. 2 and 3). Thus no information was available to suggest whether soluble nitrogen was contained largely in the protoplasm or distributed throughout the cell.

The slope of the regression line for protein nitrogen on cell volume was steeper than that for soluble nitrogen and suggested that the proportion of protein increased with cell size increase.

(*d*) *Jonathan Spot*

There was a complex relationship between Jonathan spot and breakdown which has not been reported before. While there was a negative correlation

($P < 0{\cdot}001$) between the two disorders between trees there was a positive association, i.e. more fruit with joint symptoms than would be expected by chance[*] ($P < 0{\cdot}001$), but the relation between the two disorders will not be discussed as no theory can be advanced to resolve the apparent paradox.

TABLE 3

PARTIAL CORRELATIONS OF VARIABLES

Correlation	Constant	r	Significance
Protein N per cell and cell surface . .	Mean fruit size	0·8260	$P<0{\cdot}001$
Protein N per cell and respiration per cell	Mean fruit size	0·8283	$P<0{\cdot}001$
Protein N per cell and R/P ratio . .	Mean fruit size	0·2066	N.S.
Protein N per cell and soluble N per cell	Mean fruit size	0·7414	$P<0{\cdot}001$
Protein N per cell and percentage breakdown	Mean fruit size	0·3050	N.S.
Percentage disorder and R/P ratio . .	Mean fruit size	0·2655	N.S.
Percentage breakdown and R/P ratio . .	Mean fruit size	0·1468	N.S.
Percentage disorder and mean fruit size . .	Respiration/protein	0·7795	$P<0{\cdot}001$
Percentage breakdown and mean fruit size	Respiration/protein	0·7007	$P<0{\cdot}001$
Percentage breakdown and mean fruit size	Cell volume	0·6120	$P<0{\cdot}001$
Percentage breakdown and cell volume . .	Mean fruit size	0·0943	N.S.
Respiration per cell and protein N per cell	Cell number/g	0·8225	$P<0{\cdot}001$

IV. DISCUSSION

This paper attempts to test for a wide range of crop size within a variety the indications received from the studies of different varieties at two cropping levels (Martin and Lewis 1952) and to discover something of the physiological connection between mean fruit size per tree and susceptibility to disorder (Martin 1954).

Many of the interrelations of cell attributes which were demonstrated in the first-mentioned of these papers for cell characteristics between varieties are now shown to hold for cell size differences within a variety.

[*] Calculated as follows: Expected percentage for joint occurrence on chance basis =

$$\frac{\text{Jonathan spot (\%)} \times \text{Breakdown (\%)}}{100}$$

$$\Sigma \text{ individuals}^2 \text{ (actual} - \text{expected)} = 564{\cdot}87$$

$$\text{G.T. (actual} - \text{expected)}^2 30 = 198{\cdot}18$$

Source	D.f.	Sum of Squares	Mean Square	F	Significance
G.T.	1	198·15	198·15	15·67	$P<0{\cdot}001$
Error	29	366·72	12·645		

(*a*) *Mean Cell Volume, Cell Surface, and Protein Nitrogen*

The relation between cell size and protein content has now been demonstrated for within-tree development (Robertson and Turner 1951); between varieties (Martin and Lewis 1952); and now for between-tree variations within a variety. The principle that protein synthesis keeps pace with cell enlargement is thus a characteristic of apple cells under a wide range of conditions.

Kidd *et al.* (1951), by other methods, have demonstrated a decrease in protoplasm thickness as apple cells expand. Unless their conditions were exceptional and cell size increase without accompanying protein synthesis occurred, the two results can be reconciled only by assuming that protein nitrogen becomes more concentrated in the protoplasm as the cells expand.

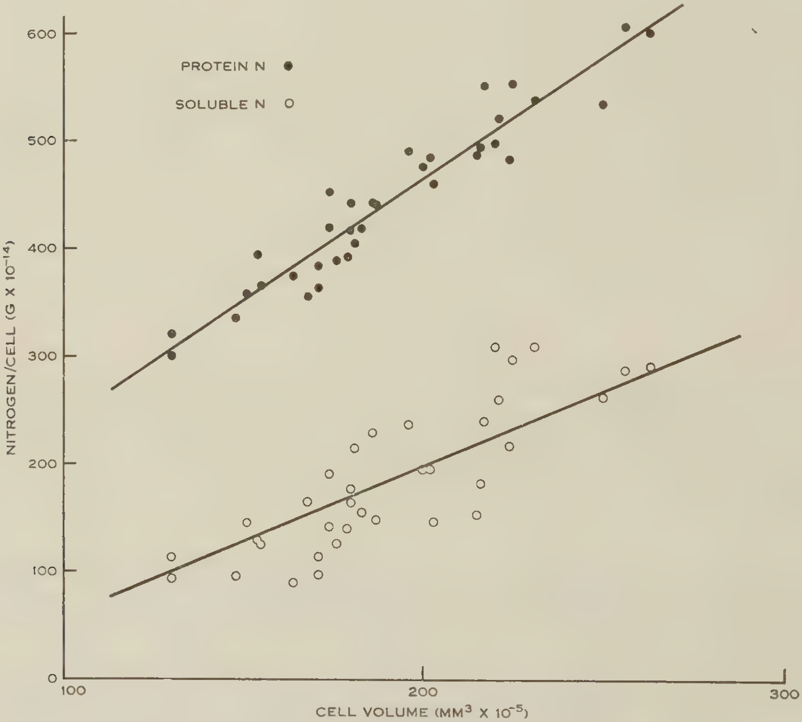

Fig. 2.—Protein and soluble nitrogen and cell volume.

(*b*) *Protein Nitrogen, Soluble Nitrogen, and Respiration*

The close relation between protein nitrogen and respiration is now shown to be a characteristic of cell growth within a variety, as well as between varieties.

Robertson and Turner (1951) suggested a "steady state" relation between protein and soluble nitrogen in their studies of cell enlargement during fruit growth. The very close correlation now shown for these two variables between trees, which remains highly significant ($P < 0 \cdot 001$) when mean fruit size per tree is held constant, supports their suggestion.

The positive correlation of protein nitrogen per cell with respiration per unit protein would be consistent with another of their suggestions, that increased protein content of cell is associated with increased difficulties of protein maintenance and respiration rate. This may be linked with the possibility of an increase in protein concentration suggested in (*a*) above.

The decline in significance of the relation between respiration per unit protein and protein nitrogen per cell when fruit size per tree is held constant may be due to the difference in level of experimental error of the different terms.

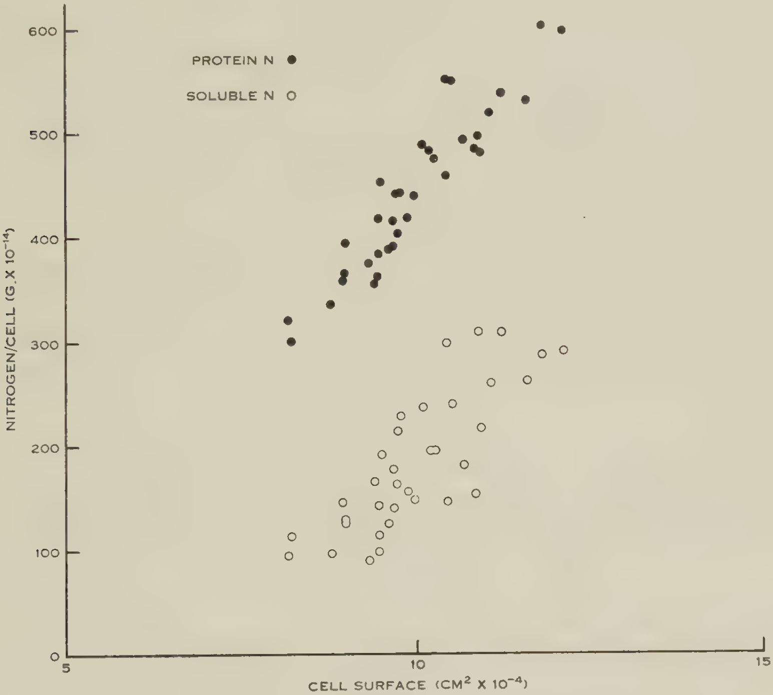

Fig. 3.—Protein and soluble nitrogen and cell surface.

(*e*) *Breakdown, Respiration per Unit Protein, and Cell Size*

The earlier suggestion that variation in disorder level might be related to difficulties of cell organization associated with cell growth has been supported by the intercorrelation of these facts. The decline in significance when mean fruit size is held constant by methods of partial correlation may be due to differences in level of precision in the different terms; the partial correlation of disorder and mean fruit size for constant respiration per unit protein may remain significant for the same reason.

If the differences in level of precision are not responsible for the magnitudes of the partial correlations, the explanation of why mean fruit size is such a good index of disorder susceptibility does not lie in the level of efficiency of respiration in cells of different sizes. However, the probability that light-crop trees not only have fewer fruit buds but may also have less cell division in the fruits in spite of their larger size, suggests weakness in cell organization that merits further study.

V. Acknowledgments

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THE EFFECT OF MOLYBDENUM ON THE ORGANIC AND INORGANIC PHOSPHORUS OF PLANTS

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[*Manuscript received March 3, 1954*]

Summary

Molybdenum, applied 2 days before harvest, had a marked effect on the phosphorus metabolism of molybdenum-deficient tomato plants grown in water-culture solutions. The molybdenum increased the concentration of organic phosphorus, with a corresponding decrease in the concentration of inorganic phosphorus. Molybdenum increased the yield of dry matter, the absolute amount of phosphorus, and the absolute amount of organic phosphorus; but did not increase the absolute amount of inorganic phosphorus. The concentration of total phosphorus did not change.

I. INTRODUCTION

Molybdenum is known to be concerned in the nitrogen metabolism of legumes and non-legumes (Mulder 1948; Anderson and Spencer 1950), and many workers have attempted to elucidate this effect of molybdenum in plant metabolism (Hewitt, Jones, and Williams 1949; Agarwala 1951; Nicholas, Nason, and McElroy 1953).

It has also been shown that molybdenum inhibits the acid phosphatases of plants (Bossard 1947; Courtois and Anagnostopoulos 1949). Acid phosphatases occur extensively in plant tissues (Ignatieff and Wasteneys 1936; Yin 1945), and have been shown to attack a wide variety of organic phosphorus compounds which are important in intermediary plant metabolism (Spencer 1954).

The present experiments were done to examine the effect of molybdenum on the distribution of phosphorus between the organic and inorganic fractions within the plant.

II. METHODS

Tomato (*Lycopersicon esculentum* Mill. var. Pan America) plants were used. These were grown in culture solutions essentially the same as those recommended by Stout and Arnon (1939), but using KH_2PO_4 instead of K_2HPO_4. The macro-elements were purified with 8-hydroxyquinoline in chloroform at a reaction between pH 1·6 and 5·6 (Gentry and Sherrington 1950). The micro-elements were all purified by repeated recrystallization. Tomato seeds, germinated on paraffined gauze placed over glass-distilled water, were transferred at the two-leaf stage to 3-l. Pyrex glass beakers. There were 20 cultures in the experiment and each culture pot held eight plants.

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TABLE 1

EFFECT OF MOLYBDENUM ON THE YIELD AND PHOSPHORUS CONTENT OF YOUNG TOMATO PLANTS

Time of Harvest	Material Analysed	Treatment	Mean Yield per Plant (mg dry wt.)	Inorganic Phosphorus (μg P/mg dry wt.)	Organic Phosphorus (μg P/mg dry wt.)	Total Phosphorus (μg P/mg dry wt.)	Inorganic Phosphorus per Plant (μg P)	Organic Phosphorus per Plant (μg P)	Total Phosphorus per Plant (μg P)
Day 0									
	Tops	Untreated	31·8	6·16	1·33	7·49	194	42	236
	Roots	Untreated	5·8	6·07	1·69	7·76	34	10	44
	Whole plant	Untreated	37·6	6·16	1·39	7·55	228	52	280
Day 2									
	Tops	Untreated	37·6	6·05	1·30	7·35	227	49	276
		Plus Mo	45·3**	4·67**	2·57**	7·24	214	114**	328**
	Roots	Untreated	6·8	5·86	2·05	7·91	39	13	52
		Plus Mo	7·5	6·90*	3·06**	9·96**	51	23**	74**
	Whole plant	Untreated	44·4	6·00	1·41	7·41	266	62	328
		Plus Mo	52·8**	5·03**	2·59**	7·62	265	137**	402**
Day 4									
	Tops	Untreated	51·1	6·09	1·20	7·29	311	60	371
		Plus Mo	67·8**	4·52**	2·66**	7·18	305	180**	485**
	Roots	Untreated	7·9	6·88	2·72	9·60	54	21	75
		Plus Mo	8·9	7·10	2·83	9·93	63	25*	88**
	Whole plant	Untreated	59·0	6·20	1·40	7·60	365	81	446
		Plus Mo	76·7**	4·85**	2·74**	7·59	368	205**	573**

* Effect of molybdenum significant at $P<0\cdot05$.

** Effect of molybdenum significant at $P<0\cdot01$.

After 3 weeks in the culture solution the plants showed the symptoms of molybdenum deficiency described by Arnon and Stout (1939). Molybdenum as sodium molybdate (=0·05 p.p.m. Mo) was applied to eight of the 20 cultures at this stage. Also, at this stage, plants from four of the cultures were harvested. Two days later, plants from four of the molybdenum-treated and four of the molybdenum-deficient pots were harvested. A similar harvest was made after a further 2 days.

Harvests were taken by cutting the plants immediately below the cotyledons. The upper part is referred to as "tops," the lower part "roots." Samples of the tops and roots were taken separately for dry-weight and phosphorus determinations.

Extracts for phosphorus estimation were prepared by grinding the plant material with cold trichloroacetic acid followed by low-speed centrifugation (Heard 1945). Inorganic phosphorus was estimated by the method of Ennor and Stocken (1950), in which the colour complex is formed under conditions which do not cause hydrolysis of labile phosphorus compounds. Total phosphorus was estimated after the extracts had been digested with perchloric and nitric acids (Piper 1944). Organic phosphorus values were obtained by difference from the values for total and inorganic phosphorus.

III. Results

The results are summarized in Table 1. Molybdenum increased the yield of the whole plants, the yield of the plant tops, but not the yield of the roots, over the short periods of 2 and 4 days.

Molybdenum altered the distribution of phosphorus between the organic and inorganic fractions within the plant. The total phosphorus per gram dry weight of the whole plants remained constant, but the concentration of organic phosphorus was increased markedly 2 days after treatment with molybdenum. The concentration of inorganic phosphorus correspondingly decreased.

The changes in the concentrations of phosphorus in the tops corresponded with those in the whole plant. The changes in phosphorus concentration in the roots did not parallel those of the tops, but were much smaller and more variable. The nature of the effect of molybdenum on the concentrations of the phosphorus fractions was the same at the 4-day harvest as at the 2-day harvest.

The addition of molybdenum caused large increases in the absolute amounts of organic phosphorus and total phosphorus in 2 days. Over this period there was no change in the absolute amount of inorganic phosphorus. The increase in the absolute amount of organic phosphorus was approximately equal to the increase in total phosphorus uptake. The increases in the absolute amount of organic phosphorus occurred in both tops and roots. The changes in the absolute amounts of phosphorus brought about by molybdenum were of the same nature, but greater in magnitude at the 4-day harvest.

IV. Discussion

The evidence presented shows that molybdenum deficiency affects the phosphorus metabolism of tomato plants. An outstanding feature of molybdenum-deficient plants was their low absolute amount and concentration of organic phosphorus.

Spencer (1954) has shown that molybdate *in vivo* inhibits the acid phosphatases of tomato. This suggests that phosphatase inhibition may be one of the metabolic functions of molybdenum in plants. It would seem therefore that the lowered level of organic phosphorus in the molybdenum-deficient tomato plants may be due to an increased activity of acid phosphatases.

There is also the possibility that all or some of the changes in phosphorus metabolism recorded in this experiment may be a result of other changes in metabolism brought about by molybdenum. An increase in organic phosphorus may possibly result from any treatment which stimulates growth through correcting a deficiency. It is perhaps of significance that in the present experiment the tops, which showed a greater increase in yield than the roots, also showed a greater increase in concentration of organic phosphorus.

In the present study it was shown that deficiency of molybdenum increased the concentration of inorganic phosphorus in the plants. Reed (1946) showed by histochemical techniques that the stems of zinc-deficient tomato plants had a much higher concentration of inorganic phosphorus than those of normal plants. Similar results with boron-deficient plants were obtained by Reed (1946), but the details were not given. No information on the effect of these deficiencies on the content of organic phosphorus was presented. The present data show that even in 2 days molybdenum increased the ratio of organic to inorganic phosphorus.

V. Acknowledgment

The author wishes to express his gratitude to Professor J. G. Wood, Botany Department, University of Adelaide, under whose guidance this work was initiated.

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THE PHOTOCHEMICAL INACTIVATION OF INDOLEACETIC ACID SENSITIZED BY NON-PROTEIN COMPONENTS OF PLANT TISSUES

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Summary

A study has been made of the IAA-destroying activity of the brei of etiolated pea epicotyls other than that due to the enzyme IAA oxidase. The protein-free dialysate sensitizes the destruction of IAA in the light but not in the dark. Blue-violet light is most effective, followed by blue-green, green, red, and yellow.

The kinetic behaviour of the whole dialysate has been studied in detail. The apparent K_s is 3×10^{-4}M and the reaction is first order with respect to IAA. The net pH optimum is 5·5. The temperature optimum is 25°C at high light intensities. At low light intensities there is no dependence on temperature over the range 8-36°C. It is concluded that there is a photochemical reaction followed by one or more dark reactions. The activity is reduced by heating or by exposing to visible or ultraviolet light. The inactivation of IAA probably involves an oxidation without the intermediate formation of hydrogen peroxide.

In the etiolated pea seedling the active material is much more abundant in the buds than in the stems, roots, or cotyledons. It is present in higher concentration in etiolated than in green pea seedlings.

The dialysate contains at least four active components. A major component depends for its activity on a heavy metal which has not yet been identified. A technique is described for the resolution and detection of the active compounds on paper chromatograms. Preliminary fractionation procedures have been devised.

I. INTRODUCTION

In recent years considerable attention has been given to the enzyme systems which produce and destroy the native auxin, indole-3-acetic acid (IAA) in plant tissues. The enzyme complex IAA oxidase has been studied in detail. It has been shown to be light-stimulated (Galston 1950; Galston and Baker 1951), although it has no absolute requirement for light.

In pure systems it has been demonstrated that riboflavin sensitizes the oxidation of IAA in the light (Galston 1949; Galston and Baker 1949*a*, 1949*b*; Galston 1950). This work has stimulated the appearance of several papers (Ferri 1951*a*, 1951*b*; Brauner 1952, 1953*a*, 1953*b*) demonstrating that a miscellany of fluorescent substances can catalyse the photo-oxidation of IAA.

This paper deals with a group of substances of low molecular weight which sensitize the photochemical inactivation of IAA and which occur in plant tissues. In the brei of etiolated pea epicotyls the magnitude of this reaction is comparable with that of IAA oxidase.

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II. Methods

(*a*) *Tissue Preparations*

Seeds of *Pisum sativum*, variety Alaska, were surface-sterilized in hypochlorite solution, washed, and soaked in tap water for 2 hr. They were sown in vermiculite in stainless-steel flats and grown in a total darkness room at 25-26°C with occasional blue light of intensity less than 1 f.c. or in a red room at 26-27°C in continuous illumination with orange-red light (Corning filter No. 348) of an intensity of 0·1 f.c.

For the preparation of breis, epicotyls of seedlings 6-10 days old were cut with scissors into an equal weight of ice-cold M/60 phosphate buffer, pH 6·6, and homogenized for 1 min in a cold Waring Blendor. The juice was strained through muslin and centrifuged in the cold for 15 min at 20,000g. The supernatant ("whole cytoplasm") was used as the source of IAA oxidase, and of the group of dialysable substances, described later, which photoinactivate IAA.

For storage in the lyophilized state, several large batches of whole cytoplasm were prepared by disintegrating epicotyls in water in an Eppenbach colloid mill at 0°C (5 g tissue per ml water), during which process the pH remained at approximately 6·5. The debris was separated in a basket centrifuge and the juice centrifuged for 15 min at 20,000g. The supernatant was then reduced to dryness under reduced pressure from the frozen state, using the lyophil apparatus of Campbell and Pressman (1944), and stored in a brown bottle over phosphorus pentoxide at 0°C. As required, the lyophilized whole cytoplasm was dissolved in M/15 phosphate buffer, pH 6·6.

(*b*) *Growth Experiments*

For growth experiments, pea seedlings were cultivated in total darkness at 25-26°C or in continuous red light at 26-27°C, according to the requirements of the experiment. Epicotyls were selected 6-7 days after sowing so that the third internodes were 1-1½ in. long. As in the method used by Galston and Hand (1949), the apical bud was broken off at the crook, the epicotyls inserted into the guillotine cutter, the distal 3 mm removed, and the subjacent 5-mm sections were cut, washed in distilled water, randomized, and used in the growth test. A single section was cut from each plant because, in preliminary experiments, when two sections were used, a bimodal frequency distribution curve for responsiveness to growth substances resulted. Test solutions contained 2 per cent. sucrose, M/150 phosphate buffer (pH 6·1), growth substance, and other addenda as stated. Approximately 20 sections were placed in each petri dish containing 20 ml of solution, and incubated in total darkness at 25°C, or 8 in. beneath a bank of three 40-W white fluorescent lights while fanned with temperature-conditioned laboratory air. Final lengths of the sections were measured after a growth period of 8-12 hr, over which time the growth rate is sensibly linear (Galston and Hand 1949).

(*c*) *Measurement of Reaction Velocity*

The reaction velocities were determined by measuring the rates of disappearance of IAA, using the modification by Tang and Bonner (1947) of

Salkowski's colorimetric method. The colour is not stable, rapidly rising to a maximum and then slowly fading. Not only the rate of development and fading but also the maximum intensity reached depend on the room temperature. In routine determinations the photoabsorption was measured when it was judged, from the room temperature and time-temperature calibration curves, that the optical density had reached its maximum. In every batch a measured time interval elapsed between adding the reagent and reading the colour of each sample, and several standards were included in each batch.

A standardized reaction system contained:

0·5 ml M/15 phosphate buffer, pH 6·6,
0·5 ml 10^{-3}M IAA,
a limiting concentration of dialysate or IAA oxidase,
addenda, and
water to a total volume of 2·0 ml.

All solutions were saturated with air. The IAA was added at zero time. For light experiments solutions were loaded into 6 by 1 in. test tubes selected for uniformity. Tubes were placed in a rack backed with aluminium paint, mounted in a fixed and reproducible position with respect to a 40-W G.E. "4500" fluorescent lamp. The solutions were approximately 2·5 cm from the surface of the lamp and received approximately 700 f.c. The light intensity of the fluorescent tube was very uniform to within 8 in. of each end. Temperature-conditioned laboratory air was fanned over tubes so that, during a 30-min run, the temperature did not vary by more than ± 0·1°C. Under these conditions the standard deviation for the measurement of the rate of photolysis of IAA was approximately 2 per cent.

III. RESULTS

Earlier workers (Tang and Bonner 1948; Galston and Baker 1951) have reported that dialysis of whole brei of pea epicotylis grown in weak red light led to a substantial increase in IAA oxidase activity, owing to the removal of a naturally occurring inhibitor. It was also observed (Galston 1950; Galston and Baker 1951) that light enhanced the activity of whole brei more than that of dialysed brei, and the suggestion was made that light accomplished this effect by "removing" (causing to dissociate) the natural inhibitor.

Wagenknecht and Burris (1950), however, found that about one-third of the activities of homogenates of bean roots or etiolated pea epicotyls was lost upon dialysis. The ash was inactive in restoring the activity. The whole dialysate was not tested.

In the author's experience, dialysis consistently diminished the rate of destruction of IAA by breis from epicotyls of pea seedlings grown either in darkness or in red light. The loss was fractionally much greater in the light than in the dark, i.e. light enhanced the activity of whole brei more strongly than that of dialysed brei. Adding back the concentrated dialysate restored the original activity. In addition it was found that the dialysate *alone* had a high capacity for destroying IAA in the light.

(*a*) *Existence of IAA-photolysing Activity of the Dialysate*

Epicotyls from 8-day, dark-grown pea seedlings were blended in a minimum volume of M/60 phosphate buffer, pH 6·6, strained through muslin, and filtered. Ten ml brei were placed in each of three cellophane bags and dialysed at 0-2°C with shaking against 10 ml, 50 ml, or two changes each of 750 ml M/60 phosphate buffer, pH 6·6. As a control, 10 ml brei placed in a test tube was also mounted on the shaker. After dialysis the volumes of brei were all adjusted to 11·0 ml. The 50 ml dialysate was frozen and the water removed by vacuum distillation, the final volume being adjusted to 11·0 ml. Residual activities of the dialysed breis were determined in the light, and compared with those of undialysed brei, of "whole" brei reconstituted by adding back the dialysate to the equivalent of its original concentration, and of the dialysate alone. Figure 1 shows that dialysis leads to a loss of activity which may be quantitatively restored by adding back the dialysate. As previously reported (Goldacre, Galston, and Weintraub 1953) this is partially attributable to a dissociable cofactor for the IAA oxidase system. However, the dialysate itself has independent activity (curve 5), of magnitude comparable with that of the IAA oxidase (curve 4).

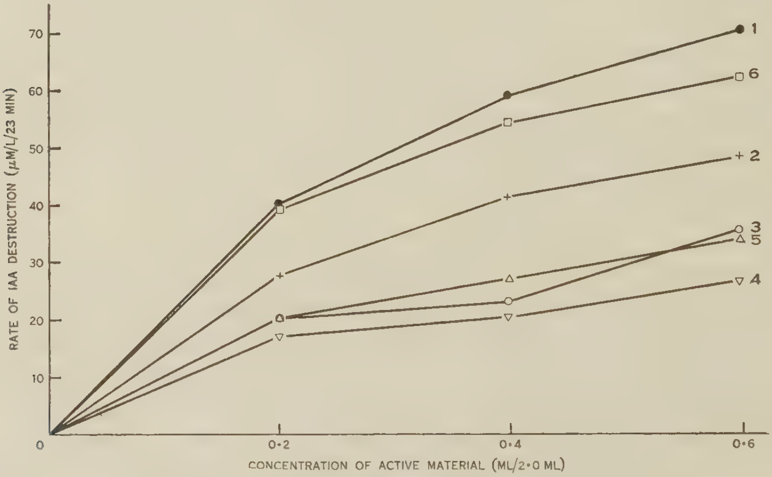

Fig. 1.—Effect of dialysis of pea epicotyl brei.
(1) Whole brei.
(2) Residue after dialysis against an equal volume of buffer.
(3) Residue after dialysis against five volumes of buffer.
(4) Residue after dialysis against 5600 volumes of buffer.
(5) Dialysate from (3).
(6) Residue plus dialysate from (3).
Initial IAA concentration 250 μM/l, temp. 26·5°C.

(*b*) *Destruction of the Biological Activity of IAA*

It is important to know whether the product of the destruction of IAA by substances in the dialysate is devoid of auxin activity. Auxin activity was determined by measuring the growth rate of pea epicotyl sections. Dialysate was prepared by dissolving 1 g lyophilized pea epicotyl brei in 5 ml M/15 phosphate buffer, pH 6·1, and dialysing overnight at 0-2°C against 15 ml phosphate buffer. Four series of petri dishes were loaded with 2 per cent. sucrose, M/100 phosphate buffer (pH 6·1), a range of IAA concentrations, in two of the series, a 1 in 20 dilution of the above dialysate, and water to a total volume of 20 ml. Sixteen epicotyl sections were floated in each dish. Two series, one with dialysate and one without, were incubated on a shaker in the dark for 10 hr at 24°C, and a similar two series were placed on a shaker 6 in. below a bank of three 40-W white fluorescent lamps, with a fan blowing laboratory air (24°C) over them. Figure 2*A* shows that, as previously reported (Galston and Hand 1949), the sections made less IAA-induced growth in the light than in the dark. Moreover, in the presence of the dialysate, growth in the light is markedly inhibited, while there is no significant effect on the growth of those sections incubated in the dark. As a control against a non-specific toxic effect of the dialysate on growth in the light (e.g. the formation of an inhibitory substance in the light) the experiment was repeated using 2,4-dichlorophenoxyacetic acid as the auxin. Growth was again reduced in the light, but in this case there was no significant effect of the dialysate in the light or in the dark (Fig. 2*B*). It may be concluded that since the growth reduction produced by the dialysate is specific for IAA-induced growth, and specific for growth in the light, the dialysate is destroying the biological activity of IAA in the light.

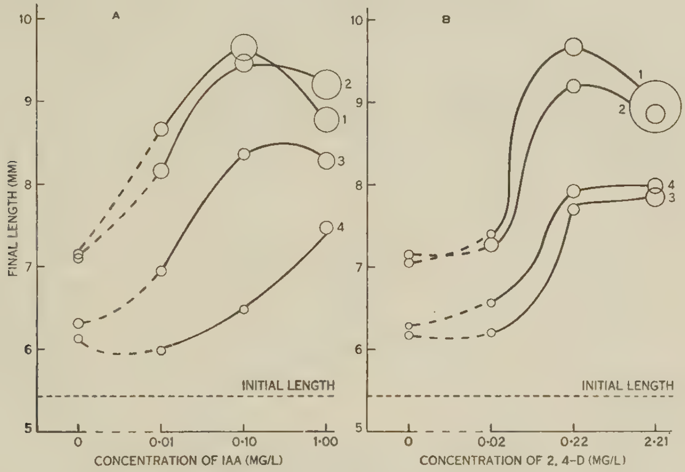

Fig. 2.—Effect of the dialysate on the growth of pea epicotyl sections in the light and in the dark under the influence of (*A*) IAA, (*B*) 2,4-D. Curve 1, dark, no dialysate; 2, dark, dialysate; 3, light, no dialysate; 4, light, dialysate. Incubated 10 hr at 24°C. Radii of circles indicate standard errors in the final lengths

Figure 3 shows the loss of biological activity of IAA as a function of the concentration of dialysate. The destruction rate increases with concentration.

(*c*) *Non-identity with Free Riboflavin*

Galston (1949) and Galston and Baker (1949*a*, 1949*b*) have described detailed work on the photosensitized destruction of IAA by free riboflavin. Since riboflavin occurs in appreciable concentration in these seedlings, it was necessary to ascertain whether the photolytic activity of the dialysate could be accounted for in terms of its riboflavin content. The dialysate was yellow in colour and fluoresced yellow-green as well as blue under ultraviolet light.

A riboflavin solution was prepared (4 μg/ml) so as to have approximately the same activity as a stock dialysate. Aliquots of the two solutions, both buffered at pH 6·6, were placed in matched thin-walled glass tubes and exposed 4 in. from a Keese ultraviolet lamp (model 909 T.P.) for 60 min. Initial and final activities were determined. Whereas the activity of the riboflavin was diminished to 28 per cent., that of the dialysate was diminished to 60 per cent. after exposure to an equal dose of ultraviolet light. This suggests that the riboflavin of the dialysate is not responsible for a major fraction of its activities, unless in the crude extract it is partially protected from destruction by ultraviolet light.

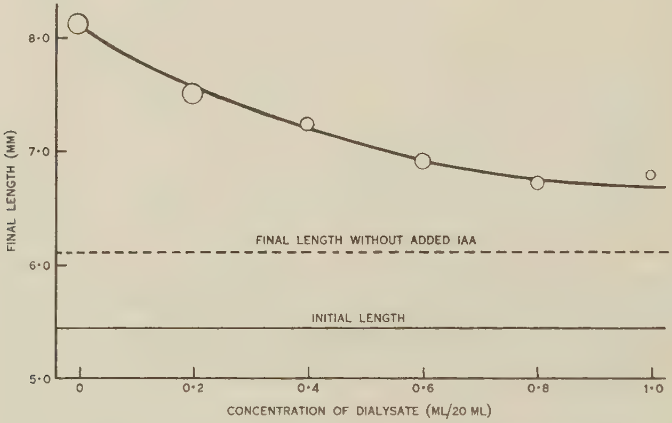

Fig. 3.—Effect of dialysate concentration on the destruction of the biological activity of IAA. Epicotyl sections incubated for 10 hr in the light at 25°C. Initial IAA concentration 0·10 mg/l. Radii of circles indicate standard errors in the final lengths.

In another type of experiment the dialysate was chromatographed alongside spots of the riboflavin solution of comparable activity. Whatman No. 1 paper was used with water as solvent. The developed chromatograms were examined under ultraviolet light. The riboflavin produced a strong yellow-green fluorescence at $R_F = 0 \cdot 33$ but the corresponding spot in the dialysate was barely discernible. Experiments to be described later, using a technique for detecting IAA-destroying activity directly on the developed chromatogram, also showed that the bulk of the activity did not correspond with the riboflavin spot. It thus became of interest to study the nature and properties of these naturally occurring substances (or substance), which are not riboflavin and which destroy IAA in the light.

(*d*) *Kinetics*

Biological catalysts are generally thought of as being proteinaceous in nature. In the above connection, however, riboflavin, a small molecule of biological origin, acts catalytically in the photodestruction of IAA (Galston 1949). The active component of the dialysate may react directly with IAA to change its identity or it may sensitize its decomposition in light. Since a catalyst might be responsible for greater activity physiologically than a substance which reacts stoichiometrically with IAA, it became an important question to determine whether the dialysate destroyed IAA in a catalytic fashion. The evidence and arguments pertinent to this question are presented in a later section after the kinetic behaviour of the system has been defined, but it may be said here that it is concluded that the dialysate reacts catalytically.

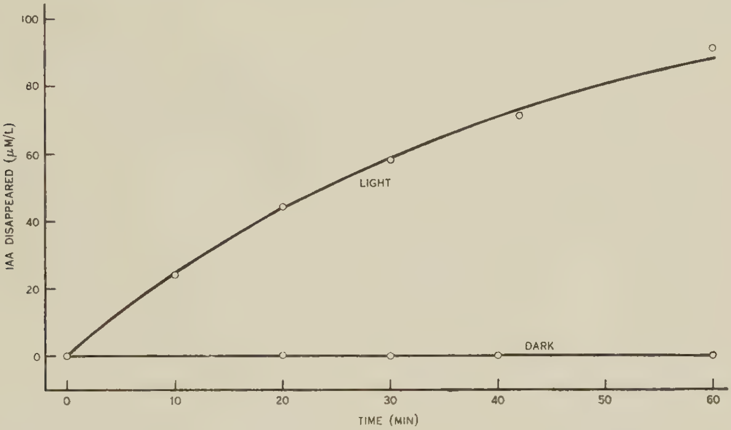

Fig. 4.—Course of IAA destruction by the dialysate, showing dependence on light. Initial IAA concentration 250 μM/l, temp. 25°C, pH 6·6.

(i) *Progress Curve.*—As a preliminary to the measurement of reaction rates, it is necessary to know the time course of the reaction under standard conditions. Two series of tubes were loaded with 0·5 ml M/15 phosphate buffer (pH 6·6), 0·5 ml dialysate, 0·5 ml water, and, at zero time, 0·5 ml 10^{-3}M IAA. One series was incubated in the light at 25°C, the other in the dark at 25°C. At time intervals, 8 ml Salkowski reagent were added to the tubes and residual IAA determined. Figure 4 shows that there is rapid destruction of IAA in the light but no destruction in the dark. With activities of this magnitude the reaction rate is practically constant for 20-30 min. In subsequent experiments, the IAA concentration change effected in this period was routinely taken as a measure of the reaction velocity, incubations all being made in the light.

(ii) *The Effect of IAA Concentration.*—The effect of IAA concentration on the reaction velocity is shown in Figure 5. The velocity is approximately proportional to concentration only below about 50 μM and the increment decreases in a hyperbolic manner, not approaching a limiting velocity even at 500 μM. Limitations of the analytical method make it impractical to exceed this value.

Let us assume for the moment that the dialysate acts as if it contains a single or a single dominant active component. According to the concept of enzyme action of Michaelis and Menten (see Wilson 1950), an enzyme and its substrate form an unstable intermediate which dissociates into free enzyme and reaction products. This premise leads to a formulation which relates reaction velocity to substrate concentration by the equation:

$$\frac{1}{v}=\frac{K_S}{V}\cdot\frac{1}{[S]}+\frac{1}{V},$$

where v = measured reaction velocity,

V = limiting velocity,

$K_S = \frac{k_2+k_3}{k_1}$, the enzyme-substrate "dissociation" constant,

$[S]$ = substrate concentration.

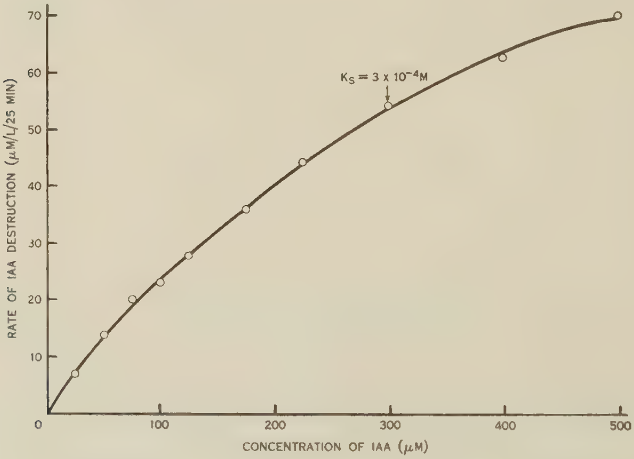

Fig. 5.—Effect of IAA concentration on the rate of its destruction by the dialysate. Temp. 25°C, pH 6·6.

By plotting $1/v$ against $1/[S]$ a straight line should result with slope equal to K_s/V and intercept $1/V$. This has been done in Figure 6 using the data of Figure 5, and the straight line fitted does not depart from the experimental data by amounts greater than can be accounted for by the expected error in the measurement of v. Thus a linear relation exists, and it may be taken that the kinetics of the dominant reaction are in accord with the concept of a catalyst-substrate intermediate complex, the dissociation constant of which from Figure 6 is approximately 3×10^{-4}M. This value has the same order of magnitude as that of IAA oxidase but the saturating concentration is much higher than that for the enzyme (Tang and Bonner 1947; Goldacre 1951*a*).

(iii) *Effect of Dialysate Concentration.*—The dialysate was prepared by dissolving 1 g lyophilized brei in 5 ml M/60 phosphate buffer, pH 6·6, and dialysing this solution against 20 ml buffer. Total reaction volume was 2·0 ml and initial IAA concentration 250 μM. The reaction velocity as a function of dialysate concentration is shown in Figure 7. Activity is proportional to dialysate concentration at low concentrations, but rapidly falls off. At high concentrations, presumably light intensity becomes limiting.

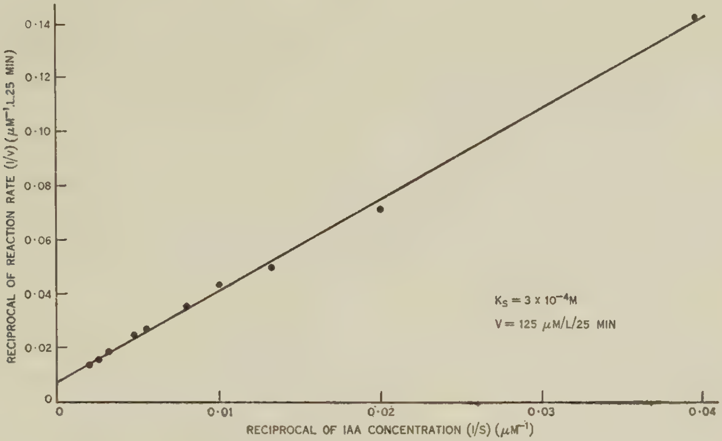

Fig. 6.—"Lineweaver-Burk" plot of the data of Figure 5.

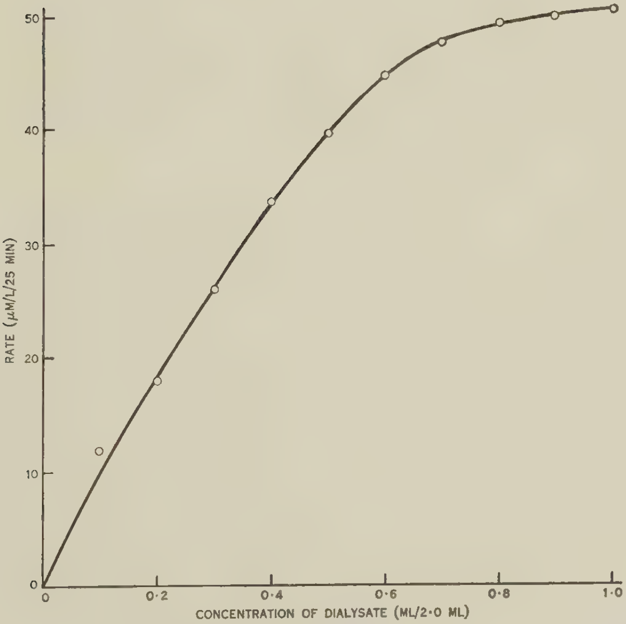

Fig. 7.—Effect of dialysate concentration on the rate of destruction of IAA. Initial IAA concentration 250 μM/l, temp. 26°C, pH 6·6.

(iv) *Effect of pH*.—A series of McIlwain's phosphate-citrate buffers was prepared to cover the range from 3 to 7 and beyond this range the pH was adjusted with 0·2N HCl or 0·1N $NaHCO_3$. Buffer (1·0 ml), 0·5 ml dialysate or 0·5 ml water, and 0·5 ml 10^{-3}M IAA were incubated in the light at 27°C for 24 min. Aliquots of 1 ml were pipetted into 4 ml Salkowski reagent and the residual IAA determined. The pH was determined on the residual reaction mixture using the glass electrode.

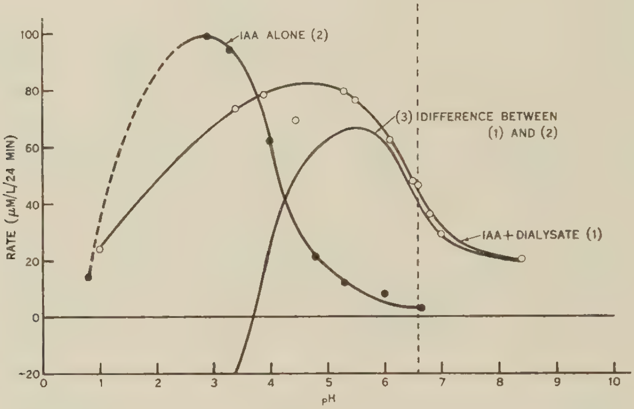

Fig. 8.—Effect of pH on the rate of destruction of IAA. McIlwain's phosphate-citrate buffers, 0·2N HCl, and 0·1N $NaHCO_3$ for extreme values. Initial IAA concentration 250 µM/l, temp. 26·5°C.

Figure 8, curve 2, shows that at acid pH IAA is destroyed by light even in the absence of dialysate. Dolk and Thimann (1932) have shown that plant growth substance isolated from *Rhizopus* culture medium, and subsequently identified as IAA, is half decomposed on standing for 3 hr at room temperature in 1N HCl in presumably low-intensity laboratory light. Algéus (1946) has also demonstrated that lowering the pH or exposing to light during storage of "pure" solutions of IAA results in losses. However, IAA itself does not absorb the visible light to which it is exposed, and its photodestruction was presumably sensitized by some pigmented impurity. All samples of IAA tested showed rapid photodestruction below pH 5, even after repeated purification (solution in NaOH and precipitation by slow addition of dilute HCl, and by recrystallization from aqueous ethanol). It was therefore necessary to carry out this control with IAA alone.

In the presence of the dialysate IAA was rapidly destroyed at considerably higher pH's (Fig. 8, curve 1). The optimum occurred between pH 4 and 5. There is little activity above pH 8.

By subtracting the smoothed curve for IAA alone from that for IAA + dialysate, curve 3 of Figure 8 is obtained. The optimum occurs at pH 5·5 and activity falls off sharply on both sides. Below pH 3·8 the activity for IAA alone is reproducibly higher than that for IAA plus dialysate. A possible explanation for this phenomenon is that, in this range, although the IAA combines more effectively with the active material of the dialysate than with the pigmented impurity in the IAA sample, the catalyst-substrate complex decomposes more slowly. Thus below pH 3·8 the dialysate partially protects the IAA from destruction by the contaminating pigment.

Curve 2 of Figure 8 shows a reproducible optimum for IAA alone between pH 2 and 3. These results with Salkowski's reagent are not in accordance with data obtained using other methods of estimation, e.g. experiments using the growth of pea epicotyl sections as a measure of residual IAA show continuously increasing destruction with decreasing pH. Thus the optimum in curve 2 seems to be a spurious effect due to incomplete specificity of the Salkowski reagent, i.e. below pH 2-3, although the biological activity of IAA is destroyed, it is converted to a product which gives a positive Salkowski reaction.

Routine determinations were carried out at pH 6·6 because the destruction of IAA in the absence of dialysate was very low there. In addition this is the pH optimum for IAA oxidase, and many previous experiments using whole brei have been carried out at this pH.

(v) *Effect of Temperature.*—The reaction velocity of a purely photochemical reaction is independent of temperature, providing that the absorption coefficient is constant over the temperature range. Experimentally it has been shown that for most reactions known to be of this kind the temperature coefficient lies between 1·00 and 1·05 (Kistiakowski 1928). When the initial photoactivation is followed by a dark reaction which derives its activation energy thermally, the temperature coefficient may be higher, and may approach that for the dark reaction when the reaction velocity of the dark reaction limits the overall rate.

The effect of temperature on the rate of photolysis of IAA was determined, somewhat crudely, for high and low light intensities in the following way. A series of 50-ml beakers containing the reaction mixtures was placed in inverted petri dish lids and backed by white glazed tile. The whole was placed in a metal trough equipped with an overflow pipe. Water from a series of temperature-controlled tanks was led by a series of rubber tubes into the petri dishes, and permitted to overflow continuously, thus water-jacketing the reaction beakers. For high light intensity a bank of three 40-W "white 4500" fluorescent lamps was suspended 8 in. above the surface of the reaction mixtures, where the intensity was 500 f.c. For low light intensity the light of the laboratory (10 f.c.) was used. Temperatures remained constant within the reaction vessels to ± 1°C.

Figure 9 shows that at low light intensity there is very little dependence of the rate on temperature. At high light intensity, between 8° and 25°C there is an increase in average rate with temperature, which becomes more marked with the time of progression of the reaction. This suggests that the initial light reaction is followed by one or more dark reactions. Above 25°C there is a rapid decline in average rate with increasing temperature, especially at longer incubation times, suggesting that the catalyst is undergoing more rapid destruction at higher temperatures.

(vi) *Effect of Light.*—As shown in Figure 4, the dialysate sensitizes the destruction of IAA only in the light. Experiments to determine the relationship between activity and absorbed light energy did not yield precise reproducible results, probably because of the crudeness of the optical systems available. It may be said, however, that within the ranges used (up to 730 f.c. incident intensity from a "white" fluorescent lamp, or up to 1600 f.c. from a tungsten "photoflood" lamp after filtering through 6 in. of water) the activity increased with increasing incident energy.

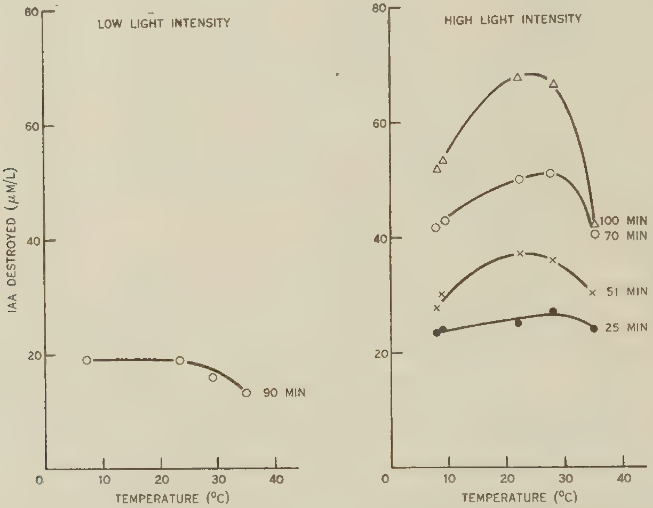

Fig. 9.—Effect of temperature on the photodecomposition of IAA by the dialysate. Initial IAA concentration 250 μM/l, pH 6·6.

A crude action spectrum was determined in the following way. A series of 5-cm dia. petri dishes was loaded with 2·0 ml M/15 phosphate buffer (pH 6·6), 1·0 ml dialysate, and (at zero time) 1·0 ml 10^{-3}M IAA. The dishes were placed inside blackened containers covered with Corning glass filters of the appropriate transmittance. The reaction vessels were set 8 in. below a bank of three "white" fluorescent lamps, and after 25 min incubation the residual IAA was determined. The relative transmitted energies from this light source through the various filters were determined by means of a blackened thermopile with a sensitive galvanometer. Table 1 shows these data. If it can be assumed that, for each wavelength, the amount of chemical change is proportional to the incident light energy in quanta, the relative destruction of IAA per quantum calculated for each wave band gives a measure of photochemical effectiveness. It may be seen from the last column of Table 1 that the relative order of effectiveness is violet > blue > green > red > yellow.

(*e*) *Stability of the Dialysate*

(i) *To Heat.*—Dialysate (0·5 ml) prepared from lyophilized whole brei was added to a series of tubes containing 0·5 ml M/15 phosphate buffer (pH 6·6)

Table 1

EFFECT OF LIGHT QUALITY ON THE RATE OF PHOTODECOMPOSITION OF IAA BY THE DIALYSATE

Corning Filter	Transmittance Maximum, (m μ)	Relative Incident Light		IAA Decomposed (μM/1/25 min)	Relative IAA Decomposed per Quantum
		Energies*	Quanta†		
Colourless glass	—	100	100	38	100
Blue-violet No. 511	380-440	9	7	17	640
Blue-green No. 428	400-540	39	34	35	271
Green No. 401	480-600	20	20	15	197
Yellow No. 352	560	73	75	18	63
Red No. 243	600-700	37	44	16	95

*Arbitrary units. Transmitted energies determined with a blackened thermopile and a sensitive galvanometer.

†An average value, using wavelength at centre of stated transmittance band. Relative numbers of quanta = relative energies × wavelengths.

and 0·5 ml water. Tubes were fitted with glass bulb condensers to avoid loss of water, placed in a hot water-bath maintained either at 80°C or 97°C, and at time intervals were withdrawn into an ice bath for rapid cooling. After equilibration to room temperature, 0·5 ml 10^{-3}M IAA was added and the resi-

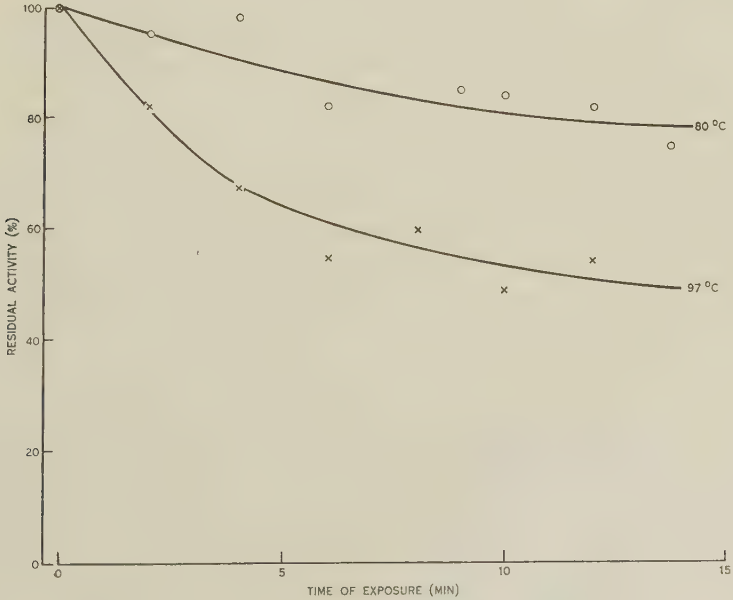

Fig. 10.—Stability of the dialysate to heat. Initial IAA concentration in assay 250 μM/l, temp. 25°C, pH 6·6.

dual activity of the dialysate was determined. Figure 10 shows that there is considerable heat inactivation of the dialysate. In 12 min approximately 50 per cent. of the activity is lost at 97°C and 20 per cent. at 80°C.

(ii) *To pH.*—Dialysate (1·0 ml) was placed in each of six beakers. The pH's were adjusted at the glass electrode over a range of values with 2N H_2SO_4 or 1N KOH, and the beakers kept in the dark at 27°C for 8½ hr. The pH's were checked, readjusted to pH 6·6, and the volumes were made up to 2·0 ml. Residual activity was then determined. Figure 11 shows that, under these conditions of incubation, the dialysate is quite stable between pH 1 and 7. Above pH 8 the activity begins to deteriorate.

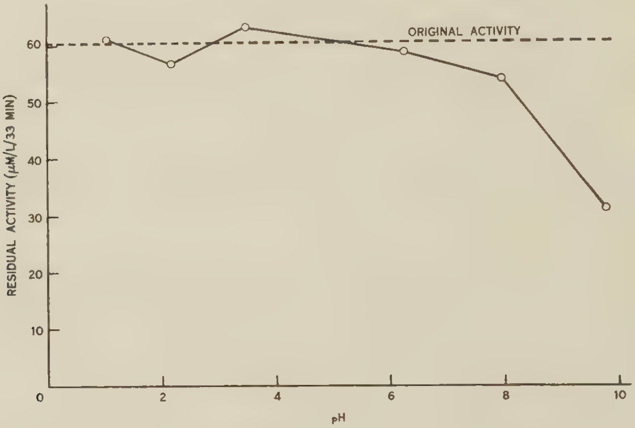

Fig. 11.—Stability of the dialysate to pH. Exposure in the dark 8½ hr at 27°C at shown pH's. Residual activity determined at pH 6·6, 23°C, and initial IAA concentration 250 μM/l.

(iii) *To Light.*—Dialysate (0·5 ml) was added to a series of tubes containing 0·5 ml M/15 phosphate buffer, pH 6·6, and 0·5 ml water. The stoppered tubes were placed on the aluminium-painted rack beneath the 40-W fluorescent lamp used in standard light experiments, and at time intervals tubes were withdrawn into a dark cupboard. Then 0·5 ml 10^{-3}M IAA was added and the residual activity of the dialysate determined. Figure 12 shows that pre-exposure to the white light produces a rapid decay in activity. The rate of decay, however, decreases rapidly with time, and the residual activity appears to approach an asymptote which, in this experiment, occurs at 50 per cent. loss of activity (or, referring to a calibration curve for activity versus dialysate concentration, approximately 70 per cent. loss of IAA-destroying units). This may be due to the dialysate containing a mixture of active components not all of which are photo-labile.

IAA affords the dialysate some protection against photolysis. Dialysate in two concentrations was incubated in the light for 5·5 hr at pH 6·6 and 25°C, with and without 500 μM/l IAA. The four solutions were then adjusted to pH 3·0 and extracted four times with water-saturated ether to remove the residual IAA. The pH's were readjusted to 6·6 and the solutions made up to volume. Residual activities of the dialysate were then determined, each at two concentrations, and compared with the original activity. Figure 13 shows that while there is great loss of activity in all cases, especially in dilute solution, the presence of IAA during the pre-exposure period reduces the loss appreciably.

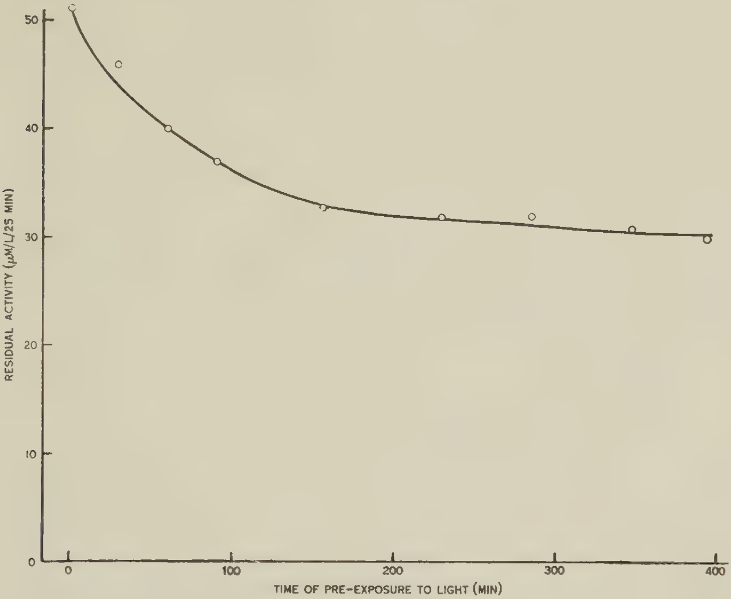

Fig. 12.—Stability of the dialysate to light (dialysate pre-exposed to 700 f.c. from a white fluorescent lamp at pH 6·6). Initial IAA concentration 250 μM/l, temp. 24°C, pH 6·6.

(*f*) *The Question of Catalyst or Reactant*

In a previous section attention was drawn to the importance of distinguishing whether the constituents of the dialysate which photoinactivate IAA act as a catalyst or a reactant. It has tentatively been accepted that the action is catalytic, and now the evidence will be considered. Useful criteria for deciding this question are the following:

(i) At the completion of a reaction, a catalyst should be recoverable with little or no loss of activity.

(ii) A reactant consumes a stoichiometric quantity of substrate so that at equilibrium the amount of substrate consumed should be directly proportional to the initial concentration of reactant. A catalyst may be expected to consume more than an equivalent number of moles of substrate.

(iii) In a simple system of reactants, the initial reaction velocity is proportional to the concentration of each reactant raised to the power of the order of the reaction with respect to each reactant, according to the collision theory of reaction rates. In a catalysed system, on the other hand, where the substrate forms an intermediate catalyst-substrate complex in accordance with the Michaelis-Menten concept, a plot of the initial rate against substrate concentration gives a rectangular hyperbola, and a plot of the reciprocals of these values gives a straight line (Wilson 1950).

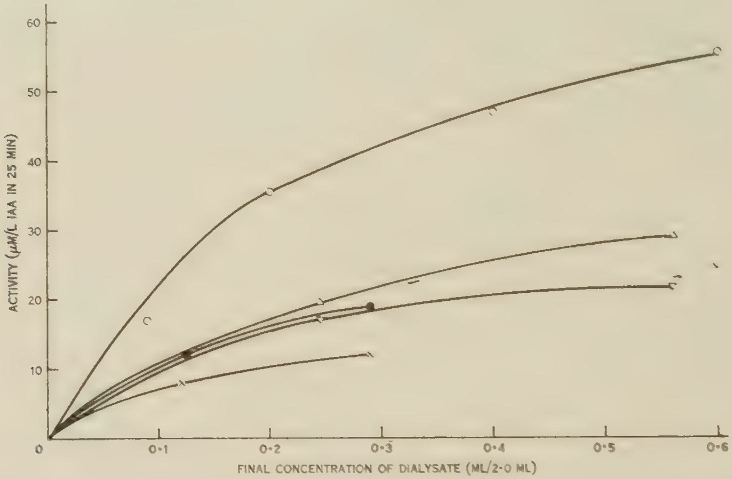

Fig. 13.—Protection of the dialysate by IAA from destruction by light. Temp. 25°C, initial IAA concentration 250 μM/l, pH 6·6. (O) Unexposed dialysate; (△) full concentration dialysate pre-exposed in presence of 500 μM/l IAA; (▽) full concentration dialysate pre-exposed in absence of IAA; (●) half concentration of dialysate, pre-exposed in presence of 500 μM/l IAA; (X) half concentration dialysate, pre-exposed in absence of IAA (see text for details).

A number of experiments has been carried out with these criteria in view.

(i) Light is essential for effecting any decomposition of IAA in the presence of dialysate. As indicated in Figure 12, the dialysate is rapidly inactivated in light so that, even in the absence of IAA, it is never possible to recover the activity quantitatively at the end of an experiment. However, referring to Figure 13, it is clear that, when irradiated in the presence of IAA, there is no greater loss in residual activity of the dialysate than when irradiated in the absence of IAA. On the contrary, IAA exerts some protective action.

(ii) It has never been possible to effect 100 per cent. destruction of IAA by prolonged incubation in the light with the dialysate preparations used, presumably because of the rapid photodestruction of the dialysate itself. In fact, in preliminary experiments a rough proportionality existed between maximum amount of IAA destroyed and the initial dialysate concentration (Fig. 14). Superficially this would seem to suggest that there is a stoichiometry between IAA used and dialysate provided. However, it may be shown that if the dialysate is pre-illuminated in the absence of IAA, then added to IAA, the rate (i.e. the slope of the progress curve) is equal to or less than that at the corresponding time interval in Figure 14. This proves that the limit is approached due to the destruction of dialysate, not by reaction with IAA but by exposure to light.

(iii) If the dialysate acted as a reactant, then the reaction velocity v should be given by

$$v = k[\mathrm{IAA}]^n[\mathrm{D}]^m,$$

where n = order of reaction with respect to IAA,

m = order of reaction with respect to dialysate constituent,

at all concentrations of IAA and dialysate. However, a linear plot of v against [IAA] and [D] in both cases results in a curve concave to the concentration axis (Figs. 5 and 7), which could only be obtained if the order of reaction were less than one.

On the other hand, a "Lineweaver–Burk" plot of activity versus IAA concentration falls closely approximate to a straight line (Fig. 8), which is in agreement with the concept of the action of a catalyst.

The most reasonable interpretation of these experiments is that the dialysate contains a photoreceptor which sensitizes the destruction of IAA in a catalytic manner.

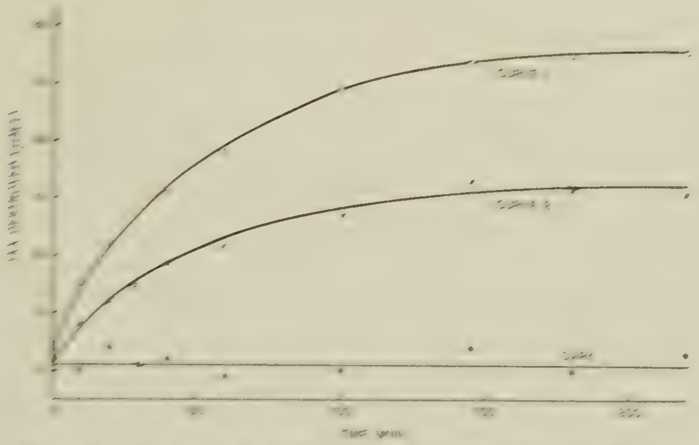

Fig. 14—Relation between maximum IAA destroyable and dialysate concentration. In curve 1, dialysate volume = 10 per cent. of reaction mixture; in curve 2, 5 per cent. Initial IAA concentration 250 µM/l, temp. 25°C, pH 6·8.

(g) The Requirement for Oxygen

The most common photosensitization reactions are oxidations (Blum 1941). It is already known that IAA undergoes oxidation catalysed by IAA oxidase, and photo-oxidation in the presence of riboflavin, and it is of interest to determine whether this dialysate-catalysed reaction is oxidative in nature.

Thunberg tubes were loaded with 1·0 ml dialysate, 1·0 ml M/15 phosphate buffer (pH 6·8), and 1·0 ml water in the main arm and 1·0 ml 10^{-3}M IAA in the side-arm. The tubes were filled with oxygen, air, or argon. At zero time the IAA was tipped in and the tubes placed in the light in the usual way. After a period of incubation aliquots were removed and residual IAA determined. This was repeated at two successive stages of purification to be described later. From Table 2 it is clear that the activity of crude dialysate shows no dependence on oxygen tension. After partial purification a relative though not absolute dependence appears. It seems that a requirement for oxygen exists, but that in the whole dialysate there is present an alternative hydrogen acceptor, in concentration high enough to account for the observed loss of IAA. The first purification step removed enough of this acceptor to make it become rate-limiting during the reaction, but did not remove all.

Table 2

EFFECT OF OXYGEN TENSION ON THE RATE OF PHOTODECOMPOSITION OF IAA BY THE DIALYSATE

Activity values are comparable along horizontal but not along vertical lines

Preparation	Incubation Time (min)	IAA Destroyed (μM/l) in		
		Argon	Air	Oxygen
Whole dialysate	26	51	54	51
Purification 1*	50	35	55	62
Purification 2†	40	49	64	70

*Whole brei was saturated with ammonium sulphate, set at 0-2°C overnight, and the precipitated proteins filtered off on diatomaceous earth. The filtrate was extracted three times into 1/20 weight of phenol. Three volumes of ether were added to the phenol solution, which was then extracted three times with 1/10 volume of M/15 phosphate buffer, pH 6·6. This aqueous fraction now contained most of the original activity and was diluted 1 in 5 before use in this experiment.

†Two ml of the above fraction were placed on a column of powdered cellulose 2·2 cm dia. by 50 cm long and chromatogrammed in water, the eluate being collected in a fraction cutter. A fraction at approximately $R_F = 0{\cdot}8\text{-}0{\cdot}9$, which was yellow in colour, fluoresced blue, was shaken into phenol and, after adding excess ether, back into 5 lots of 0·5 ml water. This was used as the active preparation.

Many photosensitized oxidations have been shown to involve the primary formation of hydrogen peroxide from oxygen and water through the action of the light-activated pigment (Blum 1941). It has already been shown that hydrogen peroxide is an intermediary oxidant in the destruction of IAA by IAA oxidase (Goldacre 1951*b*). The following experiment was carried out to determine whether hydrogen peroxide is involved in the photo-oxidation catalysed by the dialysate. Two series of dialysate concentrations were incubated in the light at pH 6·6 with 250 μM/l IAA. One series contained crystalline beef liver catalase in concentration in excess of that required to inhibit IAA oxidase completely. Figure 15 shows that catalase is not inhibitory. It may be concluded that free hydrogen peroxide is not an essential intermediate.

(*h*) *The Action of Inhibitors*

The dialysate is strongly inhibited by reagents that sequester heavy metals, e.g. KCN, H_2S, NaN_3, NH_2OH, 8-hydroxyquinoline, sym-diphenyl carbazide.

Owing to their interference in the Salkowski reaction, many of the chelating agents could be tested only at very low concentrations. As H_2S also interfered strongly, any precipitated sulphide was filtered from the dialysate through sintered glass and the H_2S was blown off with nitrogen before testing for activity.

From Table 3 and Figure 16 it is clear that a major fraction of the activity of the dialysate depends on the presence of a heavy metal. The ashed dialysate has no activity, and this observation, together with the fact that the activity is heat- and light-labile suggests that the metal may be in coordination complex with an organic moiety. The fact that activity is not second order with respect to the dialysate (Fig. 7) speaks against an interaction of an organic molecule with a substantially free ion.

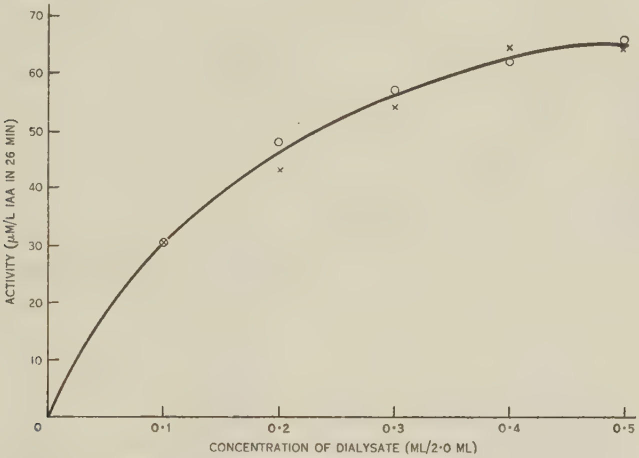

Fig. 15.—Effect of catalase on the photolysis of IAA by the dialysate. O, with catalase; X, without catalase. Initial IAA concentration 250μM/l, temp. 24°C, pH 6·6.

Albert and Gledhill (1947) have made a study of the specificities and sensitivities of a large series of chelating agents at pH 7. Of those suitable for use in the above experiment, the specificities are not such as to permit positive identification of the metal. It may be said that the metal is neither calcium nor magnesium, since 8-hydroxyquinoline inhibits but fluoride does not; nor is it copper, since benzoin oxime does not inhibit.

The addition of Cu^{++}, Co^{++}, Fe^{++}, Fe^{+++}, Mg^{++}, Zn^{++}, or Mn^{++} at 10^{-4}M concentrations to the whole dialysate produced no increase in activity. The free concentrations of native ion may already have been saturating. Cu^{++}, Co^{++}, and Mn^{++} at this concentration produced some inhibition. Attempts to remove the native metal before testing the effect of adding back these ions, were not successful.

(*i*) *Distribution of the Active Material*

(i) *In Tissues of Etiolated Pea Seedlings.*—Pea seedlings grown for 9 days in total darkness were dissected into apical buds, stems, cotyledons, and roots.

Weighed amounts of each fresh tissue were cooled and ground with sand in a measured volume of M/15 phosphate buffer (pH 6·6) in a cold mortar. Aliquots from the supernatants were placed in cellophane bags and dialysed overnight at 0°C against equal volumes of buffer. The dialysates were then assayed for activity in the usual way. Figure 17 shows that, per unit fresh weight of tissue, the apical buds have manifold greater activity than stems and roots, which have two to three times the activity of the cotyledons.

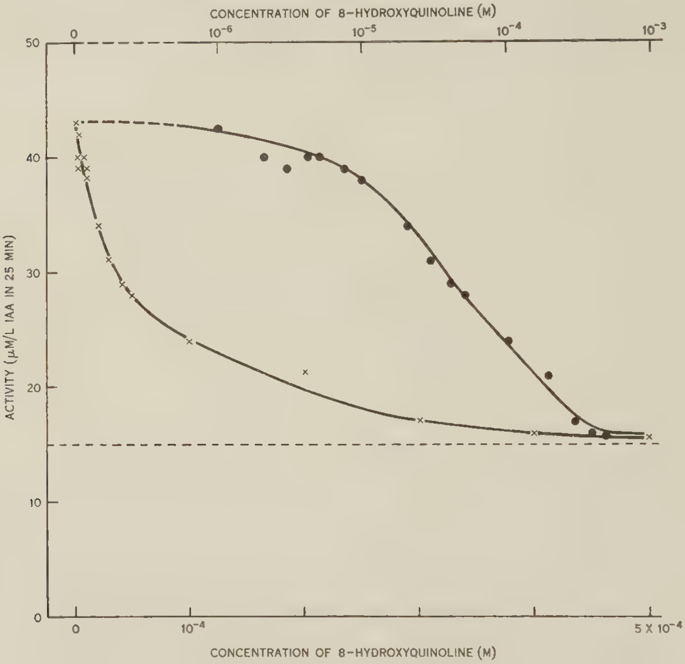

Fig. 16.—Residual activity of the dialysate as a function of concentration of 8-hydroxyquinoline. Dialysate incubated 15 min with 8-hydroxyquinoline before adding IAA. Initial IAA concentration 250 μM/l, temp. 24°C, pH 6·6. Data plotted logarithmically and arithmetically; ● = log plot, X = arithmetic plot.

(ii) *In Tissues of Green Plants.*—When a similar experiment was carried out with leaves, stems, stem tips (including leaf and stem in region where the stem had not yet elongated), and roots of pea seedlings grown for 15 days in a glass-house, substantial "apparent activity" was noted. However, subsequent experiments in which (*a*) incubations were carried out in the dark or (*b*) Salkowski reagent was added immediately after adding the dialysate to the reaction mixture, showed that only a small fraction of the "apparent activity" was in fact due to light-activated catalytic activity. The bulk of the "apparent activity" is attributed to either an interference in the Salkowski reaction or to an instantaneous dark reaction with IAA by some component of the dialysate. Similar results were experienced with green leaves of spinach, oat, and cocklebur, catalytic activity being relatively greater in younger tissue.

(*j*) *Preliminary Fractionation Behaviour*

(i) *Solubilities.*—Two-ml aliquots of dialysate, at pH 6·6, made acid with dilute HCl, or alkaline with dilute NaOH, were shaken with an equal volume of diethyl ether, *n*-butanol, light petroleum, or chloroform. The organic solvent layer was separated off, reduced to dryness under reduced pressure, and the residue taken up in 1·5 ml M/15 phosphate buffer (pH 6·6), 0·5 ml 10^{-3}M IAA was added, and activities determined in the light. In no case was any measurable activity obtained. The yellow colour and most of the fluorescence also remained in the aqueous phase.

TABLE 3

EFFECT OF INHIBITORS ON THE ACTIVITY OF THE DIALYSATE

Substance	Concentration (M)	Activity (%)	Biologically Interesting Ions Sequestered
None	—	100	
KCN	0·0125	28	
	0·0025	47	
NaN_3	0·0025	41	
	0·0005	61	
	0·0001	87	
H_2S*	Saturated*	4	Fe^{++}, Fe^{+++}, Cu^{++}, Zn^{++}, Mn^{++}, Co^{++}
NaF	0·025	98	Ca^{++}, Mg^{++}
	0·005	96	
8-Hydroxyquinoline	0·00025	62	Mn^{++}, Zn^{++}, Fe^{++}, Fe^{+++}, Co^{++}, Cu^{++}
	0·00005	72	
Benzoin oxime	0·001	106	Cu^{++}
	0·0002	98	
α,α′-Dipyridyl	0·0025	82	Fe^{++}
	0·0005	75	
	0·00025	70	
Sym-diphenyl carbazide	0·00025	63	Zn^{++}, Cu^{++}
	0·0001	73	
Diethyl dithiocarbamate	0·00001	82	Mn^{++}, Fe^{++}, Fe^{+++}, Zn^{++}, Co^{++}, Cu^{++}
	0·00002	102	
Sulphanilamide	0·0025	91	
	0·0005	96	
Hydroxylamine	0·00125	75	Fe^{++}
	0·00025	82	
Guaiacol	0·00025	41	
	0·00005	49	
$MnCl_2$	0·0025	85	
	0·0005	80	

* H_2S from a cylinder was saturated with water vapour and passed through a volume of dialysate set in a brine-ice bath in the dark for 3 hr. The dialysate was filtered through sintered glass; the H_2S was blown out with nitrogen (scrubbed with alkaline pyrogallol and water) at 0°C in the dark until the effluent gas failed to darken lead acetate paper. Residual activity was then measured.

It was possible to extract the active material of the dialysate into phenol or into acetone after saturating the aqueous phase with ammonium sulphate.

Twenty ml of dialysate plus 40 ml saturated aqueous ammonium sulphate were extracted twice with 5 g phenol. Five volumes of freshly distilled ether were added to the phenol extracts and shaken with 15 ml, then 5 ml, water. The aqueous extract was found to contain 80 per cent. of the original activity, and most of the colour and fluorescence.

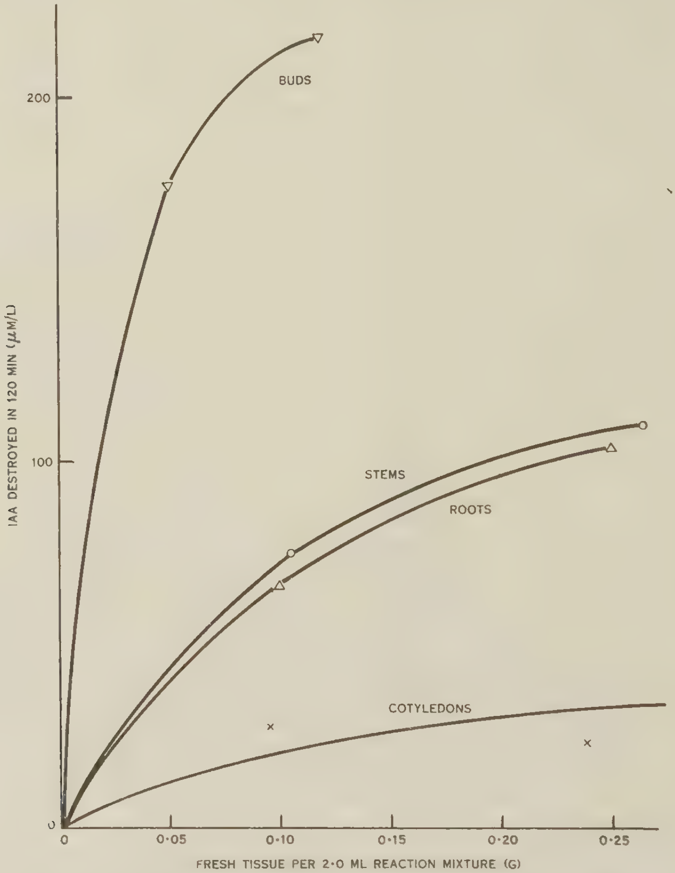

Fig. 17.—Distribution of activity of the dialysate in etiolated pea seedling tissues. Initial IAA concentration 250 μM/l, temp. 24°C, pH, 6·6.

Ten ml of dialysate were saturated with solid ammonium sulphate and extracted twice with 20 ml acetone. The acetone extract was evaporated to dryness at reduced pressure and room temperature. The residue was dissolved in 10 ml M/15 phosphate buffer (pH 6·6) and assayed for activity. About 80 per cent. of the original activity was recovered, as well as most of the colour and fluorescence.

Both extraction procedures are useful for the preliminary purification and concentration of the active material. Phenol is preferable because of the greater ease of returning the active material to the aqueous phase.

(ii) *Chromatography.*—Of the many adsorbents tested, silicic acid + "Hyflo supercel" (3 + 1) and powdered cellulose proved most useful, particularly the latter, because simultaneous runs on an analytical scale could be made on filter paper, with approximately the same relative rates of movement. The locations of active spots on filter paper were determined in the following manner. After the chromatogram was developed it was air-dried and momentarily dipped in a solution of 10^{-3}M IAA in water-saturated ether. After evaporation of the ether the papers were suspended horizontally in an atmosphere saturated with water vapour beneath a bank of fluorescent lamps. After incubating for an hour, the paper was sprayed with a modified Salkowski reagent (M/100 ferric chloride in 6 per cent. perchloric acid) and placed in a 100°C oven for 30-60 sec. The active spots appear white on a pink background.

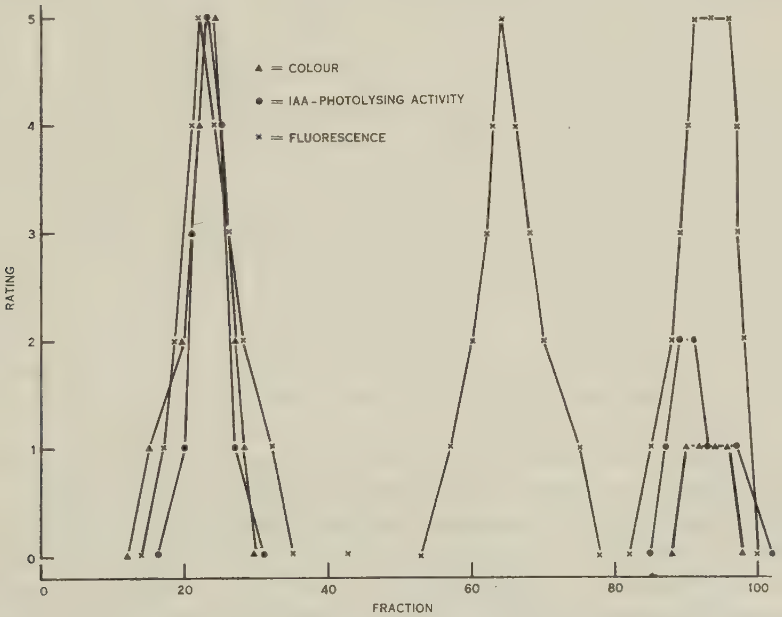

Fig. 18.—Fractionation of dialysate on a powdered cellulose column, using water as solvent. Fractions numbered, randomized, and subjectively rated 0 to 5. ▲, Colour; ●, IAA-photolysing activity; X, fluorescence.

A column 2·2 by 50 cm was filled with dry powdered cellulose, under suction, and was washed well with water. Two ml concentrated dialysate (prepared by extraction into phenol and re-extraction into water) were placed on the column, which was developed with water. The first 80 ml were discarded, then 2·8-ml fractions were collected. The fractions were numbered, randomized, and subjectively evaluated for colour and for intensity of fluorescence under ultraviolet light, each being assigned a rating from 0 to 5. The fractions were also roughly assayed for IAA-photolysing activity by placing a drop on filter paper, drying, dipping the paper into a water-saturated ether solution of IAA, incubating in the light for 1 hr, then spraying with the modified Salkowski reagent. Figure 18 shows that the IAA-photolysing activity was confined to two peaks, both of which were coloured and fluorescent. The slower-moving peak (fraction 90), which was of lesser activity, fluoresced yellow-green and, when rechromatogrammed on paper, travelled identically with riboflavin in several solvents. Although both fractions containing the pigmented bands sensitize the decomposition of IAA in visible light (Table 4), no absorption maxima could be detected in the visible region, presumably because the concentrations were too low. Fraction 22 showed a maximum at 265 mμ and a minimum at 242 mμ, but these features cannot with certainty be attributed to the active constituent.

Table 4

SEPARATION OF THE ACTIVE COMPONENTS OF THE DIALYSATE BY CHROMATOGRAPHY ON PAPER

Spot	R_F in		Fluorescence under Ultraviolet	IAA-destroying Activity
	Water	Butanol-pyridine-water (10:5:6)		
1	0·32	0·45	Yellow-green	+
2	0·65	0·35	Blue	−
3	0·85	0·1 to 0·2	Blue	++
4	0·85	0·45	—	++
5	0·85	0·70	—	++
6	0·85	0·55	Blue	−

Three similar fluorescent bands were obtained by chromatographing the original concentrated dialysate on paper, using water as a solvent. Most of the activity resided in the fastest-moving spot ($R_F = 0{\cdot}85$), none in the centre spot ($R_F = 0{\cdot}65$), and a small amount in the slowest spot ($R_F = 0{\cdot}32$), which is probably riboflavin. It was possible to further resolve the first spot by chromatographing at right angles in a solvent mixture of *n*-butanol-water-pyridine in the proportions 10 : 5 : 6 by volume. Three active spots separated, only one of which was accompanied by detectable fluorescence (Table 4). A second fluorescent spot was devoid of measurable activity. Thus the dialysate is shown to contain at least four compounds active in photolysing IAA. Only two of these are accompanied by fluorescence in the procedures used.

Attempts were made to distinguish which of these spots was the metal-containing component. Developed two-dimensional chromatograms were sprayed with 5×10^{-4}M 8-hydroxyquinoline before dipping the papers into the IAA solution, but in no case was the activity of any spot destroyed. It was not possible to put enough of the dialysate on to the paper to test after eluting. It seems that either during the process of chromatography the dissociable metal has been separated from its organic moiety or else that the reaction proceeds so rapidly on the paper that even an almost totally inhibited compound can still sensitize the destruction of IAA. Table 1 gives a crude action spectrum showing maximum activity at short wavelengths, with perhaps a second smaller peak in the red. Since this activity is a composite due to at least four substances, it would be desirable to resolve them and study their activities separately.

Brauner (1953*b*) has demonstrated that an anthocyanin (violanin) and two flavanols (viola-quercitrin and quercetagetin) are without photolytic activity toward IAA, and that an alcoholic solution of chlorophyll has slight activity.

Owing to the limitation of time, no further work has been done on the isolation of these active components.

IV. Discussion

This paper is principally descriptive in character. For simplicity in reference, many of the details of behaviour of the pea epicotyl dialysate have been discussed in the appropriate section where the result is reported.

Etiolated pea epicotyls have been shown to yield a group of at least four substances of low molecular weight which photoinactivate IAA at a rate comparable with that of IAA oxidase in the same tissue. Kinetic data indicate that the action is catalytic, and that the affinity for IAA is of the same order as that between IAA oxidase and IAA. The activity of dialysed IAA oxidase preparations is considerable in the dark and may be approximately doubled by exposure to 700 f.c. from a "white" fluorescent lamp. The dialysate has no activity in the dark, so that its light-dark differential is greater than that of IAA oxidase.

At this stage it is not possible to assign, with any degree of certainty, a physiological role to constituents of the dialysate, nor to IAA oxidase. It has been determined (Foster, McRae, and Bonner 1952) that the dissociation constant for the complex formed between IAA and the intracellular receptor site with which it reacts before evoking the growth reaction, is approximately 10^{-7}M. Since this is three orders of magnitude lower than that for IAA oxidase or the dialysate, the two latter systems would compete unfavourably against the receptors for combination with free IAA when the growth receptors are unsaturated (i.e. where a positive growth response to IAA is obtained). Thus in a tissue continuously supplied with IAA, the equivalent concentration of enzyme or of the photolysing substances of the dialysate would have to approach 1000 times that of the growth receptors in order that a change in the activity of the former would be reflected in an immediate growth response. On the other hand, in tissues where growth is limited by supraoptimal concentrations of IAA, these two systems may be expected to destroy free IAA and reduce the inhibitory effects.

It is important to consider whether the active substances of the dialysate may enter into the light-induced responses of plants known to involve changes in auxin concentration. Isolation and chemical identification of the components would permit their ready assay in the tissues and their study in purified *in vitro* systems, leading to experiments that might determine their physiological action in plant tissues.

V. Acknowledgments

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ON THE MECHANISM OF TRANSMISSION OF NON-PERSISTENT PHYTOPATHOGENIC VIRUSES BY APHIDS

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Summary

The following is a working hypothesis, based on published data, to elucidate the mechanism of transmission of non-persistent viruses by aphids: The stylets of the vector become contaminated with virus during probing in infected

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tissues. Only this virus is of significance in disease transmission, and ingested virus plays no part in the process. Some of the virus on the stylets is inactivated by salivary fluids. Viruses differ in their susceptibility to this inhibition and different species of aphids vary in the production of the inhibitor. Fasting decreases the activity of the salivary inhibitors.

Experimental evidence has been sought to determine whether virus is carried on the stylets, and whether salivary secretions are inhibitory. Although direct proof of these points is lacking, there is evidence that infective virus is carried mechanically, and that some viruses are sensitive to components of the saliva of certain insects. A study of the effects of fasting on the physiology of aphid vectors has shown that fasting has no effect on digestive enzyme formation, the rate of penetration of the stylets into the leaf, or the amount of plant material ingested. Fasting does have an effect on aphid feeding behaviour and on the production of saliva. Finally, explanations for vector specificity and differences in vector efficiency are examined.

It is concluded that there is evidence in favour of the working hypothesis.

I. INTRODUCTION

The majority of viruses causing diseases of plants are transmitted by insects. These insect-transmitted, phytopathogenic viruses fall into two main groups, designated as "persistent" and "non-persistent" viruses by Watson and Roberts (1939). The vectors of persistent viruses remain infective for long periods, and there is usually a latent period following an acquisition feed during which the virus cannot be transmitted. All the leafhopper-borne viruses and some aphid-borne viruses (e.g. potato leaf roll virus, pea enation mosaic virus, filaree red leaf virus, tobacco rosette virus, a strawberry crinkle virus, and others) belong to this category. The vectors of non-persistent viruses can transmit immediately after an infective feed, but generally remain infective for only a short period of time. This group includes the majority of aphid-borne viruses, many of which are of considerable economic importance.

The mechanism of transmission of non-persistent viruses is uncertain; Black (1954) has recently said that "our understanding of what actually occurs during aphid transmission is far from complete." This paper presents a hypothesis that appears to explain most of the data available, and gives details of experiments to test the validity of some aspects of the hypothesis.

Four mechanisms of aphid transmission of non-persistent viruses have previously been suggested. These may be termed (*a*) biological transmission (see, for example, Smith and Lea 1946), (*b*) transmission by the salivary apparatus (Bradley 1952), (*c*) transmission by regurgitation, and (*d*) mechanical transmission (Hoggan 1931, 1933, 1934). The route taken by the virus in each of these hypothetical mechanisms is illustrated in Figure 1.

There are objections to each of these suggestions, and consideration of published data on aphid transmission of non-persistent viruses led to the following working hypothesis:

The stylets of the aphid become contaminated with virus during probing in infected tissue. Only this fraction of the virus is of significance in disease transmission; some of the virus may be ingested, but upon reaching the midgut it plays no part in disease transmission. Some of the virus on the stylets is inactivated by the salivary fluids. This inactivation requires time, and after short periods sufficient still remains for active virus to be introduced into a susceptible host plant and to initiate infection during the probing by the insects' stylets. Some viruses are very sensitive to inactivation by the saliva, and once a salivary sheath is formed transmission of the diseases caused by these sensitive viruses is no longer possible through that feeding puncture. The insect produces the salivary inhibitor less readily after a period of fasting than it does if removed from one host directly to another. Furthermore, viruses differ in their susceptibility to the salivary inhibitors from different vectors.

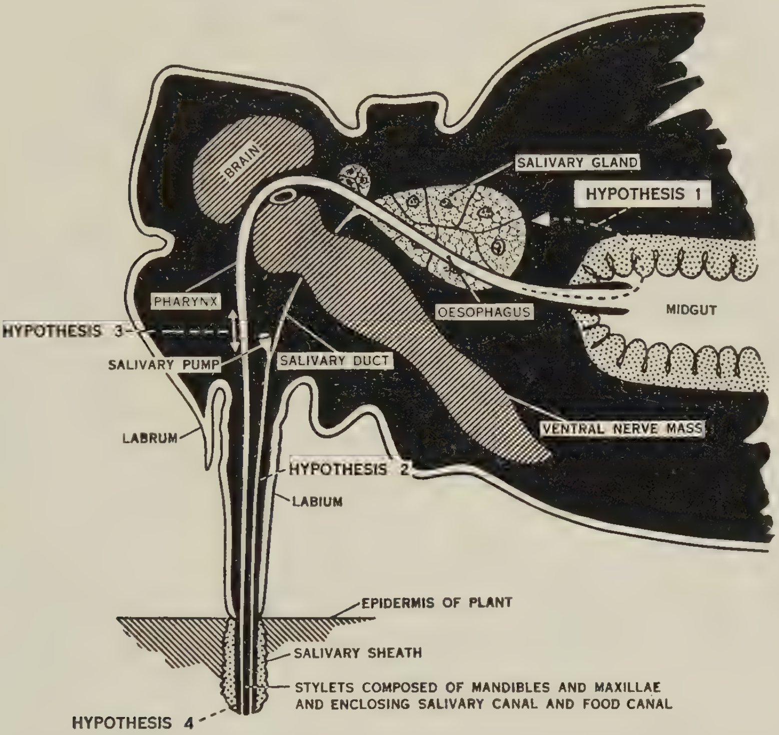

Fig. 1.—Diagram of median longitudinal section of anterior part of an aphid, showing the supposed course of virus in each of the four hypotheses of transmission. In Hypothesis I (biological transmission) the virus is thought to be ingested, to pass to the midgut where absorption occurs; the virus then reaches the haemocoele, passes to the salivary glands, and is transmitted to a susceptible host in the saliva. In Hypothesis II (transmission by the salivary apparatus) the virus is thought to contaminate the salivary canal and to be forced out at the commencement of the subsequent feeding period. In Hypothesis III (transmission by regurgitation) the virus is thought to be ingested and regurgitated during a subsequent feed. In Hypothesis IV (mechanical transmission) the virus is thought to contaminate the stylets.

Although it is basically not new, this "modified mechanical" hypothesis stresses the mechanical role of aphid vectors, towards which much criticism had been directed; but much of this criticism is vitiated by the emphasis on the inhibitory role of the saliva of the aphid.

II. Experimental Tests of the Validity of Some Aspects of the Hypothesis

Details of experiments to test some aspects of the modified mechanical hypothesis are presented in this section under the following headings:

(*a*) Is virus carried on the mouth-parts?
(*b*) Are viruses inhibited by insect salivary secretions?
(*c*) What are the effects of fasting upon the aphid vectors?
(*d*) What explanations are available for the observed specificity in virus-vector relationships?

A few negative results are mentioned to indicate certain approaches that have proved unprofitable. Data from the literature are used to corroborate the argument.

Table 1

INSTANCES OF SHORT FEEDING PERIODS THAT RESULTED IN TRANSMISSION OF APHID-BORNE VIRUSES

Thirty sec arbitrarily allowed for transference from infected plant to initiation of feeding on uninfected plant

Virus	Vector	Duration (sec) of		Aproximate Total Elapsed Time
		Acquisition Feed	Inoculation Feed	
Cauliflower mosaic	*M. persicae*	23	30	1 Min 23 sec
Cauliflower mosaic	*M. persicae*	60	22	1 Min 52 sec
Cauliflower mosaic	*B. brassicae*	60	25	1 Min 55 sec
Cauliflower mosaic	*B. brassicae*	60	60	2 Min 30 sec
Potato virus Y	*M. persicae*	28	20	1 Min 18 sec
Potato virus Y	*M. persicae*	50	15	1 Min 35 sec

(*a*) *Is Virus Carried on the Mouth-parts?*

(i) *Duration of the Transmission Cycle.*—Several authors (e.g. Sylvester 1949*b*, 1950*b*, 1952; Bradley 1952; Hamlyn 1953) have shown that the total time between the initiation of an acquisition feed and the completion of a successful inoculation feed may be no more than 1-2 min. We have confirmed this with *Myzus persicae* (Sulz.) and *Brevicoryne brassicae* (L.) transmitting cauliflower mosaic,* and *M. persicae* transmitting potato Y virus† (Table 1).

Experiments on feeding of *M. persicae* (Day and Irzykiewicz 1953) and observations on the stylets of this aphid during probing and feeding indicate that it is unlikely that sufficient material to reach the midgut could be ingested during the very short "feeds" that resulted in virus transmission. The conclusion seems inescapable that the infective virus is carried on the mouth-parts or in the anterior part of the foregut.

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* Cauliflower mosaic virus used in this paper was obtained from an infected cauliflower in Canberra. We are indebted to Mr. L. L. Stubbs, Victorian Department of Agriculture, for typing this virus.

† Potato Y virus was obtained through the courtesy of Dr. E. M. Hutton, Division of Plant Industry, C.S.I.R.O.

From the viewpoint of the modified mechanical hypothesis it is immaterial whether the virus is carried externally on the stylets or is confined to the salivary or feeding ducts as suggested by Bradley (1952). However, the salivary canal of *M. persicae* cannot have a volume much in excess of 6×10^{-11} ml (diameter approximately 1 μ, length 70 μ); on the other hand, Day and Irzykiewicz (1953) have shown that many times this volume of solution can be carried on the mouth-parts of this aphid.

(ii) *Retention of Virus Through the Moult.*—The stylets of an aphid are shed at each moult and new stylets are produced from within the head capsule. If a virus is carried on the stylets, then it could not be transmitted following a moult. This should therefore provide a test of whether virus is carried on the mouth-parts. Sylvester (1949*a*) reported examples, using *M. persicae* and beet yellow net virus, in which the virus was thought to be retained by the vector from one instar to the next. Attempts to repeat this observation with cauliflower mosaic and *M. persicae* have been uniformly negative. The experiment was performed by maintaining a culture of *M. persicae* on turnip (var. Flat Express) showing severe symptoms of cauliflower mosaic virus. Periodically the plant was searched for aphids in the process of moulting. This process occupied about 20 min and the insects were able to feed within a few minutes of casting the skin. It was imperative that specimens be removed before they had fed and that they be handled with the utmost care. Twenty newly moulted aphids were removed to turnip (var. Flat Express) indicator plants on which feeding was noted. None of these plants became infected, whereas three plants became infected when five non-moulting aphids from the same viruliferous plant were transferred to each of five test plants. From this experiment it is concluded that it is unlikely that cauliflower mosaic virus is retained by its aphid vector after the latter moults. If the modified mechanical hypothesis is correct the ability of an aphid to retain infectivity after a moult would be sufficient to exclude that virus from the category of those transmitted by the modified mechanical mechanism.

(iii) *Experiments Involving Wetting the Stylets with Virus in Solution.*—Bradley (1952) performed an experiment in which he dipped the stylets of aphids into a concentrated solution of tobacco mosaic virus, and then permitted the insects to feed upon the test plants. The absence of infection was taken as evidence that the virus was not carried upon the outside of the insects' stylets.

An attempt was made to compare the ability of *M. persicae* to transmit cucumber mosaic virus* after feeding on an infected leaf or on a concentrated solution of plant juice. Transmission after feeding on the leaf of infected spinach was readily obtained (5 out of 8). Aphids fed through a plastic membrane on sap from a similar leaf produced no infections, but this may have been due to the presence of an inhibitor in the plant (Sill and Walker 1952). Sap from infected *Nicotiana glutinosa* L. leaves was therefore clarified by low-speed centrifugation. This solution was highly infectious when mechanically inoculated on to *N. glutinosa* or spinach, but 90 *M. persicae* did not become infectious when fed through a plastic membrane on the solution for from 30 sec to 5 min and then transferred in groups to 14 spinach seedlings. It is therefore likely that the aphid is unable to pick up virus from solution as it does from the leaf. It is evident that the conclusion that the virus is not carried on the exterior of the stylets is not warranted by the results of Bradley's experiments.

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* Obtained through the cooperation of Mr. L. L. Stubbs (see Stubbs 1952*b*).

(iv) *Conclusions.*—The conclusion that the viruses are carried on the mouth-parts is thus supported by the following: (*a*) the short time between the beginning of the acquisition feed and the end of a successful inoculation feed, together with the evidence that food is not taken into the midgut during the short punctures that suffice for transmission; (*b*) the observation that the virus is not transmissible after the vector moults unless it has further access to the viruliferous host; (*c*) the comparison between the mechanism of aphid-borne viruses and behaviour of certain viruses on the mouth-parts of mosquitoes (see Day and Fenner 1953). It is pointed out that the results of experiments involving wetting the stylets with virus, or on needle transmission, do not oppose the theory that the viruses are carried on the aphids' mouth-parts.

(*b*) *Are Viruses Inhibited by Insect Salivary Secretions?*

(i) *Ingestion of Mosaic Viruses by Aphids.*—Sukhov (1944) concluded that the aphid salivary sheath is responsible for rendering the aphid incapable of transmitting tobacco mosaic virus. He was unable to detect the presence of virus in *M. persicae* feeding on virus-infected plants and concluded, on this slender evidence, that the salivary sheath acts as a filter that absorbs the virus. It has been shown that aphids contain substances that are highly inactivating against tobacco mosaic virus (Black 1939; Smith 1941), and Sukhov's inability to detect virus in the insect does not prove that the salivary sheath functions as a filter to prevent the ingestion of the virus. In fact, we have observed instances of aphids feeding through plastic membranes when the stylets protruded beyond the tip of the salivary sheath. That aphids can, in fact, ingest certain viruses was demonstrated by Severin and Tompkins (1948).

We have made a number of attempts to demonstrate that *M. persicae* ingests tobacco mosaic virus (TMV) in order to refute more directly Sukhov's suggestion. It has proved very difficult to feed aphids on plants containing a high concentration of TMV. However, it has been shown that *M. persicae*, *B. brassicae*, and *Macrosiphum euphorbiae* (Thom.) ingest TMV from solution although they produce typical salivary sheaths in the liquid. The difficulty of demonstrating the ingestion of virus even from solutions containing high concentrations of TMV clearly shows the reasons for the failure of many previous attempts. Ingestion of virus was finally demonstrated by feeding apterae of the three species for 18 hr on a concentrated TMV solution containing 5 per cent. sucrose presented through a plastic membrane. Care was taken to eliminate contamination and controls demonstrated that none occurred. The insects were then macerated in a minimum of M/15 phosphate buffer at pH 7.0. Inoculation of *N. glutinosa* with the supernatant following low-speed centrifugation caused no local lesions. However, virus could be separated from the solution by centrifuging in a Spinco ultracentrifuge for 45 min at 107,000g. The pellet was then redissolved in buffer and the solution ultracentrifuged again. The resulting pellet was taken up in buffer and the solution used to inoculate leaves of *N. glutinosa*. Local lesions of TMV were then produced, showing that the inhibiting substances from the aphids had been separated from the virus (cf. Black 1939).

In spite of the demonstration that the salivary sheath does not filter out virus particles, the modified mechanical hypothesis suggests that the saliva of the aphid is responsible for the inability of the insect to transmit the virus. Trypsin is an inhibitor of those mosaic viruses with which it has been tested, but no mechanism has been suggested which allows the insects' proteinase to come in contact with the virus; however, the virus must come into contact with the saliva of an aphid that feeds for more than a few minutes.

Although a method has recently been described for obtaining the saliva of leafhoppers (Braun and Maramorosch 1951), the minute amount produced by aphids makes it unlikely that sufficient could be collected to examine its inhibitory properties directly. An experiment was performed to determine whether the salivary sheath might contain a highly active, diffusable inhibitor. *M. persicae* in groups of 30 were permitted to feed through a plastic membrane for 6 hr upon drops of tobacco mosaic virus in phosphate buffer diluted to 10^{-4}, after which the solution was observed to contain a number of feeding tracks. The virus solution was then inoculated on to half leaves of *N. glutinosa*, the other half leaf being inoculated with a solution of virus that had remained on the plastic membranes without insects feeding on it. No differences were observed between the groups of lesions produced and it was concluded that, even though *M. persicae* is an inefficient vector of tobacco mosaic virus (Hoggan 1934), the supposed inhibitor of aphid saliva was insufficiently active to affect so large a volume of virus.

(ii) *Inhibition by Saliva from* Periplaneta.—It is fully realized that results obtained with insects unrelated to aphids are at best merely indications, but it seemed that the only approach to the problem of salivary inhibition lay in the study of insect species from which sufficient saliva could be collected to perform reproducible experiments. Saliva of the cockroach *Periplaneta americana* (L.) may frequently be obtained as droplets on the mouth-parts (Day 1951). A mixture of virus solution and cockroach saliva was used to inoculate half leaves of *N. glutinosa* (for tobacco mosaic virus) or potato (var. 11-84 of Hutton (1948) for potato Y virus) and the number of local lesions produced was compared with the number on the other half leaves inoculated with the same virus diluted with an equal quantity of water. The results are tabulated in Table 2, and demonstrate that substances in the cockroach saliva are strongly inhibitory towards tobacco mosaic and potato Y viruses.

(iii) *Inhibition by Saliva from* Nezara.—In a search for a hemipteran capable of yielding useful volumes of saliva it was found that the green vegetable bug *Nezara viridula* L. was satisfactory. The insects from the field or from laboratory colonies were mounted on microscope slides on their backs, and the stylets removed from the labium. Drops of liquid saliva accumulated at the tips of the stylets, from which it was drawn by capillarity into fine glass tubes. In this way up to 0.3 ml of *Nezara* saliva could be collected in 2-3 hr from about 100 adults. The saliva was alkaline (pH 9.5), but when mixed in equal quantities with viruses in buffered solutions the pH of the mixture was about 7·5. The technique was similar to that for *Periplaneta* saliva. The results, which are unequivocal, are given in Table 2. The saliva of *Nezara* inhibits both tobacco mosaic virus and potato Y virus.

TABLE 2

EFFECT OF INSECT SALIVA ON TMV AND POTATO Y VIRUS

Test solution, 1 part virus solution + 1 part saliva; control, 1 part virus solution + 1 part water. TMV, purified virus diluted to 10^{-4} times the concentration in tobacco leaf in M/15 phosphate buffer pH 7. Potato Y virus, 1/20 dilution of tobacco leaf infected with virus in M/15 phosphate buffer pH 7

Saliva from	Virus	Host Plant	Number of Leaves	Number of Local Lesions on	
				Test	Control
Periplaneta americana	TMV	*N. glutinosa*	8	34	277
Periplaneta americana	Potato Y	Potato (var. 11.84)	7	0	64
Nezara viridula	TMV	*N. glutinosa*	14	96	345
Nezara viridula	Potato Y	Potato (var. 11.84)	5	1	104

Differences between test and control in each experiment are highly significant.

Comparison of these results with those obtained with *Periplaneta* saliva suggests that TMV is less readily inhibited than potato Y virus; in addition, the impression was gained that *Periplaneta* saliva was more inhibitory than that of *Nezara*.

(iv) *Data on Duration of Persistence of Infectivity of Aphids.*—The above experiments demonstrate that substances capable of inhibiting certain plant viruses occur in the saliva of some insects, and suggest that viruses differ in their sensitivity to these inhibitors. This last conclusion is predicted by the modified mechanical hypothesis because of the observation that some species of aphids remain infective longer when transmitting a particular virus than do other species. Table 3 presents a summary of some of the published data on the duration of persistence of mosaic viruses in aphid vectors. Although in most instances the duration of persistence was not established within narrow limits, it will be observed that there is a wide range of variability with respect to the period during which insects remain infective, and the indications are that further work would disclose a complete series, from viruses that persist for only a few minutes to those that persist for many days. The latter have not hitherto been included in the same group of viruses. The existence of what appears to be an essentially continuous series removes one of the main reasons for separating them. It seems possible that this group of viruses comprises a series of decreasing sensitivity to the salivary inhibitors.

TABLE 3

PERSISTENCE OF NON-PERSISTENT VIRUSES IN APHID VECTORS

Times are approximate in most instances. The majority of workers have not stated at what temperatures the work was performed. Miller (1952*b*) has shown that this should be controlled in experiments dealing with persistence

Virus	Vector	Persistence		Reference
		During Feeding	During Fasting	
Cucumber mosaic	*Myzus persicae*	1-5 Min	>60 Min <120 Min	Bhargava 1951
Cucumber mosaic	*Aphis gossypii*	<20 Min	<8 Hr	Doolittle and Walker 1928
Tobacco etch	*Myzus persicae*	15 Min	>3 Hr <6 Hr	Kassanis 1941
Cabbage mosaic	*Myzus persicae*	<1 Hr	—	Kvíčala 1949
Onion yellow dwarf	*Aphis rumicis*	—	8 Hr	Tate 1940
Lettuce mosaic	*Myzus persicae*	1 Hr	>8 Hr	Kassanis 1947
Dandelion yellows	*Myzus ornatus*	1 Hr	8 Hr	Kassanis 1947
Henbane mosaic	*Myzus persicae*	Approx. 30 Min	12 Hr	Watson 1938; Watson and Roberts 1940
Potato Y	*Myzus persicae*	Approx. 20 min	12 Hr	Watson and Roberts 1940; Smith 1931
Beet mosaic	*Myzus persicae*	Approx. 3 hr	—	Severin and Drake 1948
Poison hemlock ringspot	*Rhopalosiphum conii*	≯8 Hr	—	Freitag and Severin 1945*b*
Western celery mosaic	*Aphis middletoni*	7-8 Hr	—	Severin and Freitag 1938
Pea mosaic	*Macrosiphum euphorbiae*	15 Min	Max. 24 hr	Osborn 1937*a*
Citrus quick decline	*Aphis citricidus*	>24 Hr <48 Hr	—	Costa and Grant 1951; Meneghini 1948
Clover vein mosaic	*Acyrthosiphon onobrychis*	<24 Hr	—	Osborn 1937*b*
Beet yellows	*Myzus persicae*	3 Days	—	Watson 1940
Celery yellow spot	*Rhopalosiphum conii*	≯12 Days	—	Freitag and Severin 1945*a*
Carrot motley dwarf	*Cavariella aegopodii*	Approx. 18 days	—	Stubbs 1948, 1952*a*

Evidence that the duration of persistence is influenced by feeding can be seen by comparing the persistence during continuous feeding with that during fasting, when the viruses would have less opportunity of coming in contact with a salivary inhibitor. Although the data are few (Table 3), in every instance persistence is greater in the fasting aphid than in the fed aphid. The period of survival of the virus in the feeding aphid is generally less than its survival *in vitro* (Watson and Roberts 1939). Some virus is undoubtedly wiped off the stylets during feeding, but the very short survival time of some viruses indicates that they are subjected to an additional inhibiting action. On the other hand, celery yellow spot and carrot motley dwarf viruses must be considered to be unaffected by an inhibitor.

Although most "non-persistent" viruses persist for only a short time in their vectors, Watson (1940) has shown that several plants can be infected when the vector is not permitted to feed on each for more than a few minutes. It has been demonstrated by Fenner, Day, and Woodroofe (1952) that the probability of a mosquito transmitting myxoma virus decreases with the number of feeds. A similar phenomenon has been observed with *M. persicae* transmitting potato Y virus. The experiment was performed by giving the vectors acquisition feeds of between 20 sec and 2 min and transferring them to leaves of potato (var. 11-84). The inoculation feeds were then timed with a stop-watch and the positions of feeding were marked on diagrams of the leaves. Local lesions were recorded infrequently (13 out of 321 inoculation feeds), but a significantly large proportion of the successful transmissions were produced by the first feed following the acquisition feed. Thus, nine transmissions occurred during the first feed, two during the second, one during the third, one during the fourth, and none during subsequent feeds. The rapid rate of loss of infectivity is due partly to the cleansing of the stylets during repeated feeding punctures, but it is hastened by another factor, probably the inhibiting effect of the insect's saliva. Data capable of a similar interpretation have been presented by Hamlyn (1953) who studied the transmission of cabbage black ring spot virus by *M. persicae.*

(v) *Relative Efficiency of Short and Long Acquisition Feeds.*—Further evidence of the role of the inhibitor in virus transmission should result from a comparison of the relative efficiency of short and long feeding periods on the transmission of a susceptible virus by a vector that produces an active inhibitor. This problem has been studied by several authors (Watson 1936, Kassanis 1941; Bradley 1953), although the explanation of their results has not always been apparent.

In our experiments the acquisition feeding periods of *M. persicae* were accurately timed on spinach infected with cucumber mosaic virus. "Short" feeding times were 20 sec to 2 min; "long" feeding times were 10 min to 20 min. All other factors were constant. All aphids were starved for 18 hr before the acquisition feed, and all inoculation feeds were of 15 min duration. Sixteen host plants were planted in boxes. Eight were exposed to vectors that had had short acquisition feeds and eight to vectors with long acquisition feeds, and the experiment was repeated ten times. Following "short" feeds 34 plants were infected out of 80 exposed, whereas following "long" feeds 11 plants were infected out of 80. It is concluded that "long" acquisition feeds reduce the efficiency of transmission to one-third under the conditions of the experiment.

The results are most readily explained by the hypothesis that a product of aphid feeding reduces the efficiency of transmission of cucumber mosaic virus by *M. persicae.* It is possible that the tissues tapped during short feeds contain a higher concentration of virus than those tapped during long feeds. However, in a similar test no reduction in efficiency was found with *M. persicae* transmitting cauliflower mosaic virus, suggesting that this virus is relatively unaffected by the saliva of this aphid.

(vi) *Summary of Evidence.*—It has been proved that the saliva of certain insects is inhibitory to tobacco mosaic virus and potato Y virus. Indirect evidence that aphid saliva is inhibitory is seen in the fact that some viruses persist in their vectors for shorter periods during feeding than if the vector is fasted; and by the demonstration that short acquisition feeds produce infections with greater efficiency than long acquisition feeds in certain viruses. Those viruses in which this relationship is not found are thought to be insensitive to the salivary inhibitor.

(*c*) *What are the Effects of Fasting upon the Aphid Vectors?*

Watson (1938) found that an important difference between "persistent" and "non-persistent" viruses was their reaction to fasting of the vectors before the acquisition feeding period. Fasting had two different effects on increasing the ability of aphids to transmit non-persistent viruses; one of these was ascribed to increased "appetite," but the nature of the other was not determined. The work of Bradley (1952) provided one explanation of the effects of fasting, for he observed differences in feeding behaviour following fasting. However, the cause of the second effect of fasting has not been determined.

It was considered that the solution to this problem should help to solve the larger question of the mechanism of transmission of non-persistent viruses. A study was therefore made of the effects of fasting on the physiology of the aphid, *M. persicae,* particularly on the following: (i) digestive enzyme formation; (ii) rate of penetration of stylets; (iii) amount of plant material ingested; (iv) aphid feeding behaviour; and (v) salivary sheath formation. These will be discussed in sequence.

(i) *Digestive Enzyme Formation.*—Watson (1938) and Miller (1952*a*) suggested that viruses might be inhibited by digestive enzymes produced after a period of feeding. This hypothesis received support from the reports of Weber (1928) and Miller (1932) who, on histological grounds, believed they had observed waves of secretory activity in the aphid midgut at various times after feeding. The knowledge that trypsin inhibited some viruses (Stanley 1934; Lojkin and Vinson 1931; Kleczkowski 1944) also lent support to Watson's hypothesis.

Determinations (by the azocasein method detailed by Day and Powning 1949) of the amount of proteinase present in two series of 200 *B. brassicae*, one group taken directly from the food plant and the other after 20 hr fasting, showed no difference between them. In neither series was the concentration of proteinase sufficient to cause measurable inhibition of virus activity, assuming that the efficiency of the enzyme from the aphid is not greatly different from that of vertebrate trypsin (Stanley 1934).

Watson (1938) reported that invertase was ineffective in inhibiting TMV. However, when it was found that easily measurable amounts of invertase were present in macerated *B. brassicae,* the effect of fasting on this enzyme was studied in an attempt to correlate possible changes in digestive enzyme concentration with the histological observations. Two per cent. sucrose was used as substrate and activity was measured by the iodine titration method as detailed by Day and Powning (1949). In duplicate controlled experiments no change was detected in invertase activity following 20 hr fasting. These results suggest that the effect of fasting was not related to changes in the production of digestive enzymes.

(ii) *Rate of Penetration of Stylets.*—Roberts (1940) showed the usefulness of studies on the depth of penetration by stylets in understanding the mechanism of virus transmission by aphids. However, it was apparent that a more rapid technique than that used by Roberts was necessary to permit comparison between normal and fasted aphids. It was known that the stylets remained extruded when aphids were removed from a leaf upon which they were feeding (see e.g. Bradley 1952), and it seemed likely that the stylets could be fixed in their natural position if a suitable fixing fluid could be found. This was accomplished by plunging the leaf upon which the insects were feeding into warm (50°C) Carnoy's fluid. The majority of aphids immediately separated from the leaf and the remainder could easily be detached. They were washed and immediately studied in 70 per cent. alcohol. The projecting parts of the stylets were then measured with a calibrated eyepiece micrometer, using a dissecting microscope with 15x oculars and a 7·5x objective, permitting measurements to 0·5 unit (7 μ).

To determine whether the results obtained by this method were a true indication of the depth to which the stylets had penetrated, measurements were made on 100 *M. persicae* taken directly from the *Datura stramonium* L. on which they were feeding, and the results compared with those of Roberts (1940) on the same aphid. The mean length of exposed stylets from our data was 150 μ, whereas the mean depth of penetration from Roberts' data varied from 136 to 143 μ, depending upon the food plant. The two methods thus give comparable results. It is possible that fixation contracts the labium, because all published illustrations, including those of Dykstra and Whitaker (1938) and Roberts (1940) in which fixation appears to be optimal, show a section of the stylets between the leaf and the labium that is not seen in the living condition. Portion of this, probably the greater part, is due to shrinkage of the plant tissues and so would not influence the measurements by the technique used in our experiments.

The effect of fasting on the rate of penetration was then studied by measuring the lengths of exposed stylets of aphids fasted for 10 min, 60 min, and

20 hr before various feeding periods. The results (Table 4) show (1) that *M. persicae* can penetrate as far after 30 min feeding at laboratory temperatures as they do when left to feed at will on the plants, and (2) that the differences observed in the depth of penetration following fasting were too small to account for the recorded differences in efficiency of virus transmission.

TABLE 4

MEAN DEPTH (μ) OF PENETRATION OF STYLETS OF *M. PERSICAE* INTO LEAF OF CHINESE CABBAGE AS AFFECTED BY DURATION OF FASTING

Number of stylets measured was 660. Depth of penetration of 100 *M. persicae* feeding on Chinese cabbage was 111 μ

Fasting Period	Feeding Periods (min)		
	5	10	30
10 Min	52±3*	77±3	100±3
60 Min	65±4	82±4	112±4
20 Hr	62±2	62±3	101±3

*This figure includes some insects whose stylets were not retracted following removal from the host plant (see Section (iv) below).

Efficient transmission of cauliflower and cucumber mosaic viruses was obtained after 2-min acquisition feeds, and so it was thought desirable to perform a carefully controlled experiment to determine the depth to which normal and fasted *M. persicae* can penetrate during accurately timed 2-min feeding periods on spinach. *M. persicae* starved 18-21 hr penetrated 33 μ into the leaf (minimum 0·0 μ, maximum 74 μ) whereas insects removed directly from the host plant penetrated 36 μ (minimum 0·0, maximum 80 μ) in the same period. The results demonstrate that there is no significant difference between the treatments and that there is a rapid rate of penetration during the early stages of probing. Quite frequently the aphids penetrated in 2 min through the epidermal cells into the underlying tissues.

(iii) *Effect of Fasting on the Amount of Material Ingested.*—Results of an examination of the possible effects of fasting on the amount of plant material ingested have been reported in a study of the feeding of *M. persicae* (Day and Irzykiewicz 1953). In this work radiophosphorus was incorporated in plant tissues and in artificial diets presented through plastic membranes. Although feeding periods were of necessity longer than desirable to detect possible effects of fasting, the results gave no indication that fasted insects ingested an amount different from that ingested by non-fasted aphids.

(iv) *Effect of Fasting on Aphid Feeding Behaviour.*—Bradley (1952) has shown that aphids transferred directly from one plant to another are only very rarely able to feed immediately. This is because the stylets protrude from the labium when the insect is removed from a plant on which it has been feeding and it takes time for these stylets to be ensheathed again. Until this has occurred the stylets cannot be reinserted into a plant. The normal behaviour is for the aphid then to probe the leaf lamina. It then moves off and repeats the operation until it comes to rest, generally on a vein where it feeds for a longer time. After periods of starvation the probing is frequently omitted and feeding for an extended period may occur on the leaf lamina.

It will be apparent that the time required for aphids to ensheath their stylets is important in experimental technique, because most workers have removed aphids forcibly from one plant before transferring them to a second. The number of insects with protruding stylets at various times after removal from a plant is shown in Table 5 for *M. persicae*, *B. brassicae*, and *M. euphorbiae*.

TABLE 5

DATA ILLUSTRATING THE RATE OF LABIAL EXPANSION TO COVER STYLETS AFTER REMOVAL FROM THE HOST PLANT AT 22°C

For each time 200 aphids counted, except for *M. euphorbiae*; 100 were counted for zero time and 50 after 2 min

Time (min)	Percentage With Stylets Protruding		
	M. persicae	*B. brassicae*	*M. euphorbiae*
Immediate	74·5	87·0	70·0
1	43·5	—	—
2	33·5	—	0·0
5	30·0	16·5	—
10	21·5	—	—
15	8·0	7·5	—
30	1·5	—	—

The observation that 8 per cent. of *M. persicae* and *B. brassicae* were still unable to feed 15 min after their removal from the host plant explains part of the marked increase in transmission rate reported by many authors following short periods of fasting. This explanation does not hold for *M. euphorbiae*, which is able to ensheath its stylets very readily.

One result of 30 min fasting before an acquisition feed is therefore that the insects are able to feed immediately and in many instances do so as soon as they come to rest upon a leaf.

(v) *Effects of Fasting on Salivary Sheath Formation.*—The effect of fasting described in the previous section, namely the less frequent probing by fasted aphids, can be overcome by watching the feeding behaviour of vectors with a lens. If this is done, it is found that fasting for from 30 min to 1 hr still has an effect on the ability of *M. persicae* to transmit cucumber mosaic virus. Following an acquisition feed of 1-2 min and inoculation feeds of 15 min, 40 out of 94 attempted transmissions were positive for the fasted aphids. This may be compared with 27 infections out of 96 for attempted transmissions by aphids removed directly from the food plant but which were checked for their ability to feed within 5 min of being placed on the infective plant. This increase due to fasting is low compared with that reported by previous workers because of the additional controls on feeding, but the difference between the two groups is significant. Increasing the period of fasting to 18 hr does not significantly alter the ratio of infections, viz. 24 out of 96 for fasted and 12 out of 96 for non-fasted aphids. These results cannot be used for comparison with the previous series because of differences in the source plant.

The remarkable effect of short periods of fasting (30-60 min) is readily explained by the suggestion that the inhibitor disappears from the mouth-parts or is diminished during these short periods of fasting.

These results agree with the observations of Bradley (1952), Sylvester (1950*a*), Bhargava (1951), Miller (1952*a*), and others on the effects of fasting on virus transmission. All these workers have shown a marked effect of fasting when non-fasted insects were compared with those fasted from 30 min to 1-3 hr. Fasting for periods in excess of 1-3 hr produced no further improvements in transmission. These results are in distinction to those of Watson (1938; 1946) and Watson and Roberts (1939) who reported, in all of a number of experiments, further improvement in transmission efficiency when aphids were made to fast for periods up to 24 hr. In every test the increased efficiency was slight but the probability of its being due to chance becomes very small when all experiments are considered together. A further consequence of fasting must therefore be operative. An explanation of this second effect was therefore sought.

On the hypothesis that saliva contained the virus inhibitor, it seemed possible that increased vector efficiency following long periods of fasting might be caused by resulting changes in salivary production.

Efforts were therefore made to determine the rate of production of the salivary sheath in normal and fasted insects. No sheath could be demonstrated in plant tissues by the use of Millon's reagent unless aphids (*M. persicae* on Chinese cabbage and *B. brassicae* on wild mustard) had fed for more than an hour. It was found, however, that a sheath produced in a liquid medium could be detected more rapidly. When the liquid contained very dilute methylene blue the dye was taken up by the sheath and could readily be seen. This technique permitted the observation of salivary sheaths within a minute after the plastic membrane had been punctured. This was accomplished both by non-fasted aphids and aphids fasted up to 24 hr. Starvation for 48 hr, however, resulted in feeding occurring before salivary sheaths were formed. In fact, a sheath was never seen to be formed during the first puncture after these extended periods of starvation.

This visible result of starvation suggested that shorter periods might have some effect on salivary components that was not observable by the crude method of observing salivary sheath formation. Weber (1928) has already reported marked changes in the histology of salivary glands of *Aphis fabae* L. resulting from a short feed following a period of fasting. Attempts to confirm this observation with *M. persicae* and with *M. euphorbiae* have been unsuccessful. A comparison was made of the histology of salivary glands of aphids treated in the following manner: (*a*) taken from the plant, (*b*) fasted 1 hr, (*c*) fasted 2 hr, (*d*) fasted 3 hr, (*e*) fasted 18 hr, (*f*) fasted 18 hr, fed 15 min, (*g*) fasted 18 hr, fed 1 hr, (*h*) fasted 18 hr, fed 2 hr, (*i*) fasted 18 hr, fed 3 hr, (*j*) fasted 18 hr, fed 6 hr. Careful comparison of photographs of 10-μ sections stained in Mallory's triple stain failed to reveal any differences attributable to the treatments. The periods of fasting and feeding studied should have permitted the confirmation of Weber's results, but no changes of the kind he described were noted in sections of approximately 60 insects examined. No instance was seen of cellular depletion as marked as that illustrated in Weber's Plate 12, Figure 35 (*b*). Some changes, apparently connected with a secretory cycle, were noted in the cells, but they were unrelated to the experimental treatments. It seems unlikely that *A. fabae* differs so much from *M. persicae* and *M. euphorbiae*. We conclude from our observations that the histology of the salivary glands provides no evidence of changes in the components of the salivary secretions due to fasting or feeding.

(vi) *Summary*.—The conclusions from the work reported in this section may be summarized thus: Fasting was found to have no marked effect on digestive enzyme formation, rate of penetration of stylets, or amount of material ingested. On the other hand, the demonstrable effect of fasting on feeding behaviour, stylet ensheathment, and the formation of saliva would seem to be sufficient to explain the effect on virus transmission. The hypothesis that the greatest effect of fasting is on the salivary inhibitor is supported by these results.

(*d*) *What Explanations are Available for the Observed Specificity in Virus-Vector Relationships?*

(i) *The Occurrence of Specificity*.—A review of published data makes it at once apparent that vector specificity, in the sense in which that term is applied in the leafhopper-borne viruses, does not exist in the non-persistent aphid-borne viruses. The number of examples of aphids being incapable of functioning as vectors is very small in comparison with the number of positive transmissions. *Doralis rumicis* (L.) is, however, exceptional. It has been shown to be incapable of transmitting five different non-persistent viruses. It transmitted beet mosaic once in some hundreds of experiments (Severin and Drake 1948) and transmitted western celery mosaic in only 2·2 per cent. of tests (Severin and Freitag 1938); these performances indicate an unusual inability to act as a vector and suggest that this species would repay careful study. A comparable, though less marked result was reported by Watson and Roberts (1939) for *M. euphorbiae* (= *M. gei*). They stated that "*M. gei* is a poor vector because its capacity for inactivating the viruses is greater," and their final conclusion was that "the relative efficiency of the vectors varied with the different viruses, indicating that their degree of success depended upon several interacting factors . . . " one of which is "the capacity of the vector for inactivating the virus."

Although vector specificity is not a feature of the transmission of non-persistent viruses, there are marked differences in the efficiency of various vectors. Such differences have been reported many times and some examples are summarized in Table 6.

TABLE 6

RELATIVE EFFICIENCY OF VARIOUS SPECIES OF APHIDS IN THE TRANSMISSION OF SOME NON-PERSISTENT VIRUSES

Virus	Aphis Species in Order of Vector Efficiency	Reference
Cucumber mosaic	*M. persicae* > *M. circumflexus* > *M. euphorbiae*	Watson and Roberts 1939
Henbane mosaic	*M. persicae* > *M. circumflexus* > *M. euphorbiae*	Watson and Roberts 1939
Cauliflower mosaic	*M. pisi* > *A. apii* > *M. circumflexus* > *C. capreae* > *M. solani* > *A. apigraveolens* and others	Severin and Tompkins 1948
Beet mosaic	*A. apigraveolens* > *A. apii* > *A. gossypii* > *C. capreae* > *A. pomi* > *B. brassicae*	Severin and Drake 1948
Beet mosaic	*M. persicae* > *M. solani* > *A. apii* > *M. circumflexus*	Sylvester 1952
Pea mosaic	*M. persicae* > *M. euphorbiae* = *D. rumicis*	Chamberlain 1936
Lettuce mosaic	*M. pelargonii* > *M. persicae*	Dias 1951

It should be stressed that in many instances the number of successful transmissions was insufficient to permit too rigid comparisons, but it is clear that *M. persicae* is frequently one of the most efficient vectors in addition to being one of the most common vectors. It is not, however, invariably the most efficient vector. These differences between vectors require explanation.

(ii) *Effect of Host Plant on Salivary Sheath Formation.*—Stylet tracks made by *M. persicae* in a series of different host plants were observed after staining in Millon's reagent (for the method, see Day, Irzykiewicz, and McKinnon (1952)). In *Datura*, carrot, and tomato (not a preferred host) the sheaths were fine and hairlike, but in potato the sheaths were much more conspicuous and there was a conspicuous darkening of cell walls in the vicinity of the puncture, with a noticeable increase in tissue destruction. This observation lends support to the view that the aphids react differently with respect to saliva production to different host plants.

The related problem of visibly different feeding tracks being produced by several aphid species attacking a single host plant was studied by comparing the salivary sheaths of *M. persicae* and *B. brassicae* on Chinese cabbage. Well-marked differences in the tracks were readily discernible although the differences were not as striking as those resulting from feeding tracks formed by a series of leafhoppers on *Malva* (Day, Irzykiewicz, and McKinnon 1952).

(iii) *Specific Differences in Aphid Behaviour.*—Comparisons of feeding behaviour of *M. persicae* and *B. brassicae* on swede turnips showed that the duration of each prefeeding probe was much greater for the latter than it was for *M. persicae.* A comparison was then made between the feeding behaviour of *M. persicae* and that of *M. euphorbiae.* Non-fasted *M. cuphorbiae* do not feed readily when transferred to a new host, and may remain in a feeding position for 30 min without puncturing the leaf. They generally probe more often before settling to feed than does *M. persicae*; also they probe more deeply but withdraw their stylets very much more easily (see Table 5). These readily observable differences in behaviour suggest that the efficiency of virus transmission may differ from one species to another for this reason alone. It seems probable that the "vector-host plant compatibility factor" of Simons and Sylvester (1953) is explicable by these differences in behaviour of aphids on various food plants.

A comparison was made of the ability of *M. persicae* and *M. euphorbiae* to act as vectors of cucumber mosaic virus. Watson and Roberts (1939) found that *M. persicae* transmitted this virus 20 times in 105 attempts, whereas *M. euphorbiae* transmitted twice in 105 attempts. In our experiment an attempt was made to eliminate the effect of the differences in behaviour mentioned in the previous paragraph. Aphids were starved overnight. The acquisition feed was carefully timed and watched with a 10x magnifier. Only insects that fed for 2 min were used. Those that did not obtain their acquisition feed within 5 min of being placed on the infected plant were discarded. Transmission was to spinach seedlings in the four-leaf stage. Half the seedlings in flats containing 16 plants were exposed to *M. persicae* and half to *M. euphorbiae,* and the test was repeated eight times. *M. persicae* transmitted 21 times in 64 attempts, whereas *M. euphorbiae* transmitted five times in 64 attempts. Thus *M. euphorbiae* was four times less efficient as a vector of cucumber mosaic virus than *M. persicae,* in spite of attempts to provide optimum conditions for transmission.

The modified mechanical hypothesis suggests that these differences in efficiency of transmission may be largely accounted for on the basis of differences in the salivary inhibitors, but other factors mentioned in the previous paragraphs may also be operative.

(iv) *Summary.*—It will be apparent from the above discussion that the occurrence of specificity and differences in vector ability are not arguments against the hypothesis that non-persistent viruses are transmitted mechanically. There are, in fact, many possible theoretical explanations for most of the examples of specificity observed, and there is evidence for some of these possibilities.

III. Discussion

The results of the experiments described in the preceding section tend to substantiate the modified mechanical hypothesis of the mechanism of transmission of aphid-borne non-persistent viruses advanced in the Introduction. Where the experimental evidence is inadequate there is very considerable difficulty in performing the crucial experiments, owing to the small size of the aphid stylets and attendant difficulties. No results have been obtained that conflict with the hypothesis.

In 1940 Watson and Roberts contended that the hypothesis of mechanical transmission rested on only two arguments, both of which they believed were capable of an alternative explanation. Since then additional arguments have become apparent, and the modified mechanical hypothesis is now based on the following observations: (1) The short transmission cycle, (2) the absence of a latent period, (3) the short duration of retention of the virus and the loss of infectivity in successive inoculation feeds, (4) the absence of vector specificity, (5) the ease with which these viruses can be transmitted mechanically, and (6) the absence of retention of the virus following a moult.

These six points taken together seem to permit of no explanation other than that the viruses are transmitted mechanically.

(*a*) *Difficulties in Application of the Hypothesis*

However, certain difficulties still remain; Bawden and Kassanis (1947) found that some individuals of a colony of *Myzus ornatus* Laing could transmit potato Y virus, whereas most members of the colony could not. It is possible that the few vectors produced less salivary inhibitors than the majority of the colony, but apart from the role of chance, no other explanation capable of experimental examination can be offered for this report. The report is, however, not as unusual as may at first appear. The low efficiency of some vector species found by many workers is probably attributable to the same phenomenon.

Hoggan (1931) found that certain species of aphids transmitted tobacco mosaic virus from tomato to tomato, but the same species were unable to transmit from tobacco to tobacco, even though this plant had a higher virus content than the tomato. The result has never been confirmed, but, if substantiated, may possibly be explained on the basis that the aphids produce more salivary inhibitor in response to one species of food plant than they do to another. Alternatively the virus may be less aggregated and hence more accessible in the tomato, for Black, Morgan, and Wyckoff (1950) have demonstrated that TMV may be greatly aggregated in infected tobacco.

Another report not easily explained is that of Sylvester and Simons (1951) who found that the green peach aphid is a better vector of *Brassica nigra* virus when fed on mustard (*Brassica juncea*) but that the false cabbage aphid is a more efficient vector when fed on pak choi. This interesting observation could be accounted for if each species of aphid produced different amounts of saliva or salivary components on the two hosts.

The inefficiency of the transmission of TMV and potato virus X by aphids requires explanation, because these viruses are easily transmitted mechanically and have in fact been transmitted by biting insects (Walters 1952). They may be more susceptible to salivary inhibitors than most non-persistent viruses, but this seems unlikely in view of the results reported above on *Nezara* saliva inhibition of TMV. Although both rod and spherical-shaped viruses are aphid-transmissible, TMV and potato X virus are somewhat longer than most. It may thus be difficult for them to attach to the aphid stylets. Furthermore, both have a marked tendency towards end-to-end aggregation. This aggregation occurs *in vitro*, and also in the host cell, and may add to the difficulty of acquisition by aphids. There are thus several possible reasons for the failure of TMV and potato X virus to be transmitted by aphids and this failure is doubtful evidence for discounting the modified mechanical hypothesis.

(*b*) *Re-evaluation of Classification of Aphid-borne Viruses*

Watson (1946) suggested that a basis for separating aphid-borne viruses into two groups might be their response to preliminary fasting, but pointed out the difficulties inherent in the use of this and similar criteria. On the basis of the suggestions in the present paper it is desirable to find alternative terms for what now appear to be the logical groupings of aphid-borne viruses and the terms "vector-direct" and "vector-latent" are suggested.

When a virus is transmitted on the mouth-parts, it is generally readily sap-transmissible, fasting the vector usually increases its efficiency, there is never a long latent period in the vector, and the virus does not multiply in the vector. Many such viruses reach relatively high concentrations in the mesophyllic tissues and cause mosaic diseases in plants. Those belonging to this group are vector-direct viruses. When viruses are transmitted by the mechanism involving ingestion, passage through the gut, into the haemocoele, and reinjection with the saliva, there is often a long latent period between the acquisition feed and a successful inoculation feed; many of these viruses are specific to a relatively few species of vectors and they are generally not sap-transmissible, fasting the vector usually has no effect on its efficiency; some of these viruses are confined to the vascular tissues of the host plants, they may multiply in their vectors and they may be designated vector-latent viruses. This view of aphid transmission brings these virus-vector relationships into line with mosquito-transmitted viruses. In these, rabbit myxoma is a typical vector-direct virus, whereas yellow fever is the classical example of a vector-latent virus.

There is no fundamental objection to the suggestion that a vector that normally transmits by the biological mechanism may occasionally contaminate its mouth-parts with infective virus, and so cause transmission. In some instances in which the virus is resistant and infective when introduced into the epidermal or subepidermal tissues it is possible that the vector habitually transmits by both mechanisms; but, so far, no example of this twofold mechanism of transmission is known.

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PHYSIOLOGICAL STUDIES ON THRIPS IN RELATION TO TRANSMISSION OF TOMATO SPOTTED WILT VIRUS

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Summary

The hydrogen ion concentration of the midguts of larval and adult *Thrips tabaci* and *T. imaginis* is between pH 5·0 and 5·6. The oxidation-reduction potential at these values is between + 0·184 and + 0·262 V. There is thus no difference between thrips that are vectors and those that are not vectors of the virus causing tomato spotted wilt. Furthermore, the pH and Eh conditions in the midgut of larval *T. tabaci* are unsuitable for long survival of the virus.

The larval midgut of *T. tabaci* is less thoroughly tracheated than that of the adult. The larval *T. tabaci* ingests a smaller amount of plant tissue than the adult. This quantity averages about $8{\cdot}0 \times 10^{-5}$ mg per insect per minute, when the insects were exposed to the leaf for a 30-min feeding period. The entire epithelium of the midgut of larval and adult *T. tabaci* absorbs iron and copper ions.

These data are discussed in relation to theories to account for the ability of larval and the inability of adult *Thrips tabaci* to transmit tomato spotted wilt virus.

I. INTRODUCTION

A peculiar feature of the transmission of tomato spotted wilt virus is the fact, originally discovered by Bald and Samuel (1931) and independently by Linford (1932), that the virus must be acquired by the thrips when in the larval stage. The virus may be transmitted by the adults produced from larvae that have fed on an infected plant, but not by thrips that fed on infected plants only after they had reached the adult stage. This has been confirmed by Smith (1932) and Moore (1933).

Various suggestions concerning the mechanism of this inability of the adult thrips to acquire the virus have been put forward. Smith (1932) suggested that the anatomy of the adult and larva may be sufficiently different to account for the failure of the adult to transmit; Linford (1932) considered it possible that the virus might be digested by the adult but not by the larva; and Bawden (1950) suggested that the adult gut may be impermeable to the virus. None of these suggestions has been tested experimentally. However, Sakimura (1947) concluded that larvae and adults feed on the same tissues of the plant and considered that the differences between the mouth-parts of the larvae and adults were insufficient to account for the differences in viral transmission.

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Tomato spotted wilt virus is rapidly destroyed by oxidation (Best 1939*a*). This fact, together with the observed correlation between midgut tracheation and the oxidation-reduction potential (Eh) in the insect gut (Day 1951), suggested that the virus might be destroyed in the gut of the adult thrips but not in that of the larva. It seemed worth while, therefore, to determine whether there was any difference between the pH and Eh conditions in the gut of the larval and adult thrips.

The amount of material ingested by larval and adult thrips and permeability of the midgut to iron and copper ions were also investigated.

II. Methods

A culture of *Thrips tabaci* Lind., a vector of tomato spotted wilt, was maintained on onion plants in the laboratory throughout the year. *Thrips imaginis* Bagn., a non-vector species, was collected from rose hips or dandelion flowers as required.

Midgut tracheation was studied by the methods of Hagmann (1940) and Wigglesworth (1950). Both were suitable but not all specimens were satisfactory whatever method was used.

The pH and Eh of the gut were determined by feeding about 10 thrips on each of a series of indicators in 5 per cent. sucrose solutions. Sakimura and Carter (1934) had shown that thrips would feed through a plastic membrane, and the solutions in plastic containers were therefore separated from the insects by the commercially available plastic membranes used by Day and Irzykiewicz (1953). The insects were generally left to feed overnight, but occasionally a period of 3 days was necessary when insufficient indicator was ingested during the shorter period. They were then dissected in saline and the presence of the indicators was determined in the isolated midgut. The presence of indicators that were colourless or yellow was determined by adding appropriately buffered solutions to the drop of saline in which the dissection was performed, to produce a more readily detectable colour. Similarly colour changes of oxidation-reduction indicators were checked in alkaline saline by the addition of potassium persulphate as an oxidizing agent.

The amount of material ingested was examined by feeding thrips on solutions containing ^{32}P; for details of the technique, see Day and Irzykiewicz (1953).

Absorption of iron and copper was studied by the methods of Waterhouse (1940) and Waterhouse (1945) respectively.

The tomato spotted wilt virus used consisted of a mixture of strains cultured in Bonny Best tomato plants.

III. Observations

(*a*) *Eh and pH of Midgut Contents*

The hydrogen-ion concentrations of the midgut contents of the larval and adult *T. tabaci* and *T. imaginis* were determined by the experiment detailed in

Table 1. It will be observed that the pH was between 5·0 and 5·6 for larvae and adults of both species.

Similarly, an approximate value for the oxidation-reduction potential of the midgut contents was determined by the use of the indicators listed in Table 2. Again there was no detectable difference between larvae and adults and between the vector and non-vector species. In all, the potential lay between a minimum of + 0·184 and a maximum of + 0·262 V.

TABLE 1

THE pH OF CONTENTS OF MIDGUT OF *THRIPS TABACI* AND *THRIPS IMAGINIS*

Indicator	*Thrips tabaci*		*Thrips imaginis*	
	Adults	Larvae	Adults	Larvae
Thymol blue	>2·2 <8·6	>2·2 <8·6	>2·2 <8·6	>2·2 <8·6
Bromphenol blue	>4·3	>4·3	>4·3	>4·3
Phenol red	<7·4	<7·4	<7·4	<7·4
Bromthymol blue	<6·5	<6·5	<6·5	<6·5
Bromcresol purple	<6·0	<6·0	<6·0	<6·0
Chlorphenol red	<5·6	<5·6	<5·6	<5·6
Bromcresol green	>5·0	>5·0	>5·0	>5·0
pH range	5·0-5·6	5·0-5·6	5·0-5·6	5·0-5·6

(*b*) *Tracheation of Midgut*

Examination of tracheal impregnations of larval and adult *T. tabaci* showed that tracheation of the midgut was not particularly thorough. There were no anastomoses, as in the cockroach midgut (see Day 1951), and by no means every epithelial cell was tracheated. It was apparent, however, that the adult midgut was more completely tracheated than that of the larva.

These observed differences could not be correlated with a difference in oxidation-reduction potential of midgut contents. Undoubtedly the small size of these insects contributes to the maintenance of the relatively oxidizing conditions in the midgut contents in the absence of a highly developed tracheal supply.

(*c*) *Quantity of Material Ingested by Larval and Adult Thrips*

Observation had suggested that larval thrips were responsible for greater tissue damage to host plants than adult thrips. It was thought that differences in amount ingested might possibly explain differences in the transmission of tomato spotted wilt. The following experiments demonstrated that this suggestion was unfounded.

Larval and adult *T. tabaci* were fed on 5 per cent. sucrose solution containing ^{32}P. The duration of feeding was observed through a transparent plastic membrane and an accurate estimate of the rate of ingestion could therefore be made. The insects were starved for from 30 to 60 min before being permitted to feed, and in view of the short time of feeding excretion of ^{32}P was probably very low in these instances. The results (Table 3) show that adults ingested more than larvae, and that the amount ingested by larvae averaged $1\cdot2 \times 10^{-3}$ and by adults $2\cdot0 \times 10^{-3}$ cu. mm. per insect in 1 min.

Table 2

THE OXIDATION-REDUCTION POTENTIAL OF THE CONTENTS OF THE MIDGUT OF *THRIPS TABACI* AND *THRIPS IMAGINIS*

Indicator	E'_0 of Indicator (V)		Colour of Indicator		Colour of Indicator in Gut of Thrips			
					Thrips tabaci		*Thrips imaginis*	
	pH 5·0	pH 5·6	Oxidized Form	Reduced Form	Adults	Larvae	Adults	Larvae
Indigo disulphonate	−0·010	−0·045	Blue	Yellow	Blue	Blue	Blue	Blue
Indigo trisulphonate	+0·032	−0·004	Blue	Yellow	Blue	Blue	Blue	Blue
Indigo tetrasulphonate	+0·065	+0·029	Blue	Yellow	Blue	Blue	Blue	Blue
Methylene blue	+0·101	+0·066	Blue	Colourless	Blue	Blue	Blue	Blue
Thionine	+0·138	+0·106	Blue-purple	Colourless	Blue-purple	Blue-purple	Blue-purple	Blue-purple
Toluylene blue	+0·221	+0·184	Blue	Colourless	Pink*	Pink*	Pink*	Pink*
1 : Naphthol-2-sodium sulphonate-indo 2,6-dichlorphenol	+0·262	+0·223	Blue in alk. red in acid	Colourless	Colourless	Colourless	Colourless	Colourless

*In spite of the anomalous colour, it was shown that the dye was present in the midgut in the oxidized form.

Lower figures were obtained for insects feeding on a leaf of Chinese cabbage (*Brassica chinensis* L.) for 30 min (Table 4), because the insects did not feed for all of the time and possibly also because some of the isotope was excreted. Adult female *T. tabaci* weighed about $2\cdot8 \times 10^{-2}$ mg, and ingested roughly 17 per cent. of their weight per hour. The Chinese cabbage leaf was made radioactive by standing in White's nutrient solution containing ^{32}P for 24 hr. Again, adults ingested more than larvae, but the differences between larvae and adults were clearly unrelated to inability of the adult thrips to acquire virus.

(*d*) *Midgut Permeability*

Data to test Bawden's (1950) suggestion that the adult midgut might be impermeable, but that the larval gut could be penetrated by the virus, were sought by two methods.

(i) An attempt was made to puncture the midgut with a fine steel needle sharpened under a magnifier on a hard Arkansas oilstone. The operation was performed on 188 adult *T. tabaci*, held under carbon dioxide anaesthesia, that had fed for 24 hr on tomato plants infected with tomato spotted wilt virus. After 24 hr 20 adults remained alive on the infected tomato plant. These were transferred to six seedlings of *Datura stramonium* and seven seedlings of tomato. Eight adults survived on eight of these test plants for 14 days but no plant became infected with the disease.

TABLE 3

RATE OF INGESTION OF 5 PER CENT. SUCROSE SOLUTION BY ADULTS AND LARVAE OF *THRIPS TABACI*

Insect Stage	Feeding Time (min) (*a*)	Ingested ^{32}P (counts/min) (*b*)	^{32}P Content in 1 cu. mm. Sucrose (counts/min) (*c*)	Rate of Sucrose Ingestion per Insect (cu. mm./min) $\left(\frac{b}{a \times c}\right)$	Mean Rate of Ingestion of Solution (cu. mm./insect/min)
Adult	6·25	72	9,600	$1{\cdot}2\times10^{-3}$	$2{\cdot}0\times10^{-3}$
Adult	7·50	272	9,600	$3{\cdot}8\times10^{-3}$	
Adult	17·50	534	12,600	$2{\cdot}4\times10^{-3}$	
Adult	16·00	97	12,600	$0{\cdot}5\times10^{-3}$	
Adult	15·50	440	12,600	$2{\cdot}3\times10^{-3}$	
Larva	11·00	81	9,600	$0{\cdot}8\times10^{-3}$	$1{\cdot}2\times10^{-3}$
Larva	5·33	63	9,600	$1{\cdot}2\times10^{-3}$	
Larva	8·66	128	12,600	$1{\cdot}2\times10^{-3}$	
Larva	14·00	99	12,600	$0{\cdot}6\times10^{-3}$	
Larva	5·00	111	12,600	$1{\cdot}8\times10^{-3}$	
Larva	17·75	388	12,600	$1{\cdot}7\times10^{-3}$	

(ii) Any physiological difference in absorption between the larval and adult thrips midgut would be of interest. An interesting system in the larval blowfly midgut concerns the absorption of ions of copper and iron (Waterhouse 1940, 1945). The second method of studying midgut permeability was therefore to feed solutions containing increased iron and copper salts through plastic membranes to larval and adult *T. imaginis* and *T. tabaci* for 48 hr. The dissected midguts were then examined after application of the appropriate histochemical tests for detection of these metals. All cells of the midgut stained evenly in larvae and adults of both species and no region appeared to be especially concerned with the absorption of these metallic ions.

There was thus no evidence from these experiments that differences existed between the permeability of the midgut of larval and adult thrips.

IV. Discussion

The suggestion that variation in pH may determine the distribution of virus of tomato spotted wilt in various tissues of the plant has been made by Best (1939*c*); there were logical reasons for expecting that pH, Eh, or both would be concerned in determining the distribution of the virus in the insect vector. The evidence presented above proves that there are no differences in pH or oxidation-reduction conditions in the midguts of adult or larval thrips or of a species of thrips incapable of acting as a vector of the virus, and that the explanation of the inability of the adult *T. tabaci* or of adult and larval *T. imaginis* to acquire the virus must be sought elsewhere.

Table 4

RATE OF INGESTION OF LEAF MATERIAL BY ADULTS AND LARVAE OF *THRIPS TABACI* WHEN LEFT ON ^{32}P LEAF FOR 30 MIN

Insect Stage	Feeding Time (min) (*a*)	Ingested ^{32}P (counts/min) (*b*)	^{32}P Content in 1 mg Leaf Tissue (counts/min) (*c*)	Rate of Ingestion of Leaf Tissue per Insect per Min (mg/min) $\left(\frac{b}{a \times c}\right)$	Mean Rate of Ingestion of Leaf Tissue (mg/min)
Adult	30	376	67,400	$1 \cdot 9 \times 10^{-4}$	$1 \cdot 0 \times 10^{-4}$
Adult	30	97	67,400	$0 \cdot 5 \times 10^{-4}$	
Adult	30	86	67,400	$0 \cdot 4 \times 10^{-4}$	
Adult	30	240	67,400	$1 \cdot 2 \times 10^{-4}$	
Adult	30	123	67,400	$0 \cdot 6 \times 10^{-4}$	
Adult	30	149	67,400	$0 \cdot 7 \times 10^{-4}$	
Adult	30	102	67,400	$0 \cdot 5 \times 10^{-4}$	
Adult	30	454	67,400	$2 \cdot 2 \times 10^{-4}$	
Adult	30	186	67,400	$0 \cdot 9 \times 10^{-4}$	
Adult	30	241	67,400	$1 \cdot 2 \times 10^{-4}$	
Larva	30	95	67,400	$0 \cdot 5 \times 10^{-4}$	$0 \cdot 6 \times 10^{-4}$
Larva	30	261	67,400	$1 \cdot 3 \times 10^{-4}$	
Larva	30	104	67,400	$0 \cdot 5 \times 10^{-4}$	
Larva	30	56	67,400	$0 \cdot 3 \times 10^{-4}$	
Larva	30	83	67,400	$0 \cdot 4 \times 10^{-4}$	
Larva	30	130	67,400	$0 \cdot 6 \times 10^{-4}$	
Larva	30	136	67,400	$0 \cdot 7 \times 10^{-4}$	
Larva	30	127	67,400	$0 \cdot 6 \times 10^{-4}$	
Larva	30	61	67,400	$0 \cdot 3 \times 10^{-4}$	

More surprising is the demonstration that the conditions of pH and Eh in the gut of the larval *T. tabaci,* which is capable of acquiring the virus of tomato spotted wilt, are quite unsuitable for the maintenance of the virus. Thus Best

(1939*b*) has shown that infectivity of tomato spotted wilt is greatly reduced at pH 5·1 and it is surmised from his data on oxidative destruction of tomato spotted wilt virus (Best 1939*a*) that an oxidation-reduction potential of $> +0{\cdot}100$ V would result in fairly rapid destruction of the virus. However, it is appreciated that measurement of this potential does not necessarily indicate the oxidizing power of the midgut contents. Nevertheless, the results suggest the possibility that the tomato spotted wilt virus may be protected from destruction in the midgut of the larval thrips and that such a protecting mechanism may be absent from the midgut of the adult. However, an attempt to test this hypothesis gave no support to the suggestion.

Two possible mechanisms remain to explain the differences between the ability of larval and adult thrips to acquire the virus of tomato spotted wilt from infected plants. There may be differences between the midgut permeability of the two stages, or the virus may not be able to multiply in the tissues of the adult, whereas it finds suitable conditions in the larva.

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THE BLOOD SOURCES OF SOME AUSTRALIAN MOSQUITOES

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Summary

The results of over 1400 precipitin tests on the stomach contents of engorged mosquitoes to determine the source of their blood meals are recorded. Fifteen species of mosquitoes are included in this survey, but three species, *Anopheles annulipes, Culex fatigans,* and *C. annulirostris,* dominated the collections.

Engorged mosquitoes have been collected from three types of situation.

(i) Restricted animal habitats such as human dwellings, fowl houses, horse stables, dog kennels, and rabbit warrens;

(ii) The general farmyard environment; and

(iii) River flats and creek banks.

The collections from restricted animal habitats have indicated that for the species under consideration the site of collection is indicative of the blood source of the mosquitoes therein collected. Farmyard collections have given valuable information on the range of hosts attacked by nocturnally active mosquitoes. River flat collections have been most useful in disclosing rabbit-feeding species. Collections have also been made of mosquitoes attracted to man in the field and a small but significant percentage of these have been found to be already engorged. Such collections have been most interesting in that species feeding on marsupials have been revealed.

To the information derived from these precipitin tests has been added our accumulated observations of biting activity in the field, on man and other animals. In this way it has been possible to prepare a statement of the range of blood sources known to be utilized by each species and to give some indication of the preferences exhibited by certain mosquitoes for particular blood sources.

Anopheles annulipes has been shown to feed particularly on cattle and rabbits although other animals, man, and poultry are also attacked. *Culex fatigans* bites man in the domestic environment but is equally attracted by fowls in fowl-runs, or by horses in stables or dogs in kennels. Biting by this species is almost exclusively under shelter. *C. annulirostris* attacks man and most domestic animals but is most active out of doors. Rabbits are frequently attacked by this species. It is well known as biting man but man is not a preferred blood source. *Culex pipiens australicus* has been shown to attack rabbits and poultry. Species which have been revealed as attacking marsupials are *Anopheles stigmaticus, Aedes notoscriptus, A. alboannulatus,* and *A. queenslandis.*

Finally the significance of the feeding habits is discussed in relation to possible disease transmission by the various species. *Anopheles annulipes, Culex annulirostris,* and *C. pipiens australicus,* because of the frequency with which they attack rabbits, have an important role in myxomatosis transmission. *Culex fatigans* and *C. annulirostris,* attacking both poultry and man, are likely suspects for the transmission to man of Murray Valley encephalitis. *Anopheles annulipes,* on the other hand, because it attacks man only casually, is unlikely to assume any importance as a vector of malaria.

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I. INTRODUCTION

Little precise information has so far been available as to the usual sources of the blood meal for the majority of Australian mosquitoes, nor has existing knowledge of the range of hosts attacked been adequately reported. On only one previous occasion have precipitin tests been used to determine blood sources of wild-caught mosquitoes in Australia. This was in the studies undertaken by Dr. G. A. M. Heydon on north Queensland and New Guinea anophelines and reported under the species concerned in Lee and Woodhill (1944).

The present work has been based on precipitin test determinations of species of mosquitoes, both anopheline and culicine, found in southern Australia, and has been stimulated by the need for knowledge of rabbit-feeding species in connection with myxomatosis transmission, and of the range of hosts utilized by mosquitoes likely to be in any way involved in the cycle of transmission of Murray Valley encephalitis.

Initially, collections of mosquitoes, obviously blood fed, were made from typical animal habitats, houses, stables, poultry runs, and the like, in order to determine whether the site of collection had any relation to the source of the blood meal. Later these collections were extended to areas where one would be unable to presume the source of a blood meal owing to the variety of animals available. Typical of such areas were farmyards and the banks of rivers and creeks. Finally it was also found possible to collect engorged mosquitoes attracted to man in the field. Here again it was scarcely possible to even guess the blood source.

Over 1400 precipitin tests have now been carried out and the results of these tests are reported in full in Table 1. In all, tests of the blood meals of 15 species of mosquitoes are incorporated although only three species have been collected in large numbers.

II. METHODS

Blood-fed mosquitoes for precipitin tests were usually captured in suction tubes. As far as possible, collections have been made in the morning, so that digestion would not have proceeded too far, but afternoon collections representing the previous night's feeding activity have still yielded quite useful results. After collection the gut contents were smeared with a needle on to filter paper, and identification of the mosquito was either made simultaneously or, if not obvious, the insect was pinned for later examination. The filter paper strips with dried blood spots (usually sun dried) were stored in glass bottles over silica gel and as soon as possible placed in a refrigerator for storage. The actual precipitin tests were then carried out from a fortnight to over 12 months later.

In preliminary tests antisera were obtained from various sources, but it was soon found expedient and more satisfactory to prepare our own antisera, and stocks of antisera for man, ox, sheep, horse, dog, marsupial (*Trichosurus*), rabbit, fowl, duck, and lizard (*Varanus* and *Tiliqua*) have been prepared. With few exceptions species specificity has been maintained. Anti-fowl sera proved relatively non-specific and gave reactions with the blood of duck, rosella (*Platycerus eximius*), and dove (*Streptopelia suratensis*). Anti-duck, however, appears to be specific at least to the degree that it does not react with fowl, rosella, or dove. As expected, the anti-possum serum is more correctly an anti-marsupial serum. The anti-goanna serum has not been as non-specific as we hoped as it has proved unsuitable for the blood of the water lizard (*Sphenomorphus quoii*), the water dragon (*Physignathus leseurii*), and the blue-tongue (*Tiliqua scincoides*). Similarly, an antiserum prepared from the blue-tongue failed to react with the other species.

All antisera have been prepared in rabbits, with the exception of anti-rabbit, for which hens have been used. A variety of methods have been employed in preparing the various antisera. In the early work the intravenous method described by Arnold *et al.* (1946) was followed, but later Proom's (1943) technique was exclusively used. This involved the intramuscular injection of 10 ml of alum-precipitated serum of the appropriate animal at 30-day intervals until a satisfactory titre has been obtained. This was usually after the second injection. Testing for titre, or bleeding for antiserum preparation, is carried out 12-14 days after each injection. At the commencement of the work it was decided that titres of no less than 1 in 8000 were required for this type of testing and in the majority of cases it has been found possible to maintain or better this standard.

Individual tests have been carried out in open capillary tubes of 1·3 mm bore and 0·75 mm wall thickness. The pattern followed has usually been to test each blood spot against appropriate antisera for the one or two most likely blood sources and only when negative results are thus obtained to test against the range of available antisera. In this way the procedure has been less complicated and a considerable economy in the use of antisera has also resulted. From time to time known blood spots have been included with the unknown series as a check against any breakdown in the specificity of the antisera in use.

It is considered that, for the tests reported, positive results have been recorded for the majority of suitable spots collected. In early collections rather high (up to 50 per cent.) negative results have been obtained, but, as experience indicated that certain types of spots seldom gave a positive reaction, later collections were more selective, and positive results approaching 100 per cent. of the total collected were recorded. Actually, of the total, 84 per cent. have given positive reactions and very little significance is attached to the 16 per cent. negative results. Most of these negatives did come from blood-fed mosquitoes but at too late a stage of digestion for a positive reaction to be recorded. Admittedly we have been faced with series giving consistently negative results, and for these we strongly suspect that the range of antisera is not sufficiently wide, but these particular tests have not been included in the detailed results recorded below. They will, however, be mentioned again at a later stage.

III. DETAILS OF PRECIPITIN TESTS

Table 1 gives the results obtained from the present series of blood-fed mosquitoes. It has been arranged, not chronologically, but in increasing order of complexity of the environments from which the test mosquitoes have been collected, with the exception of a few limited series appearing at the end of Table 1.

IV. DISCUSSION OF RESULTS RECORDED IN TABLE 1

The localities from which these mosquitoes have been collected are all in south-eastern Australia, with the exception of a very few from Moree in north-western New South Wales and Texas in southern Queensland. Those from Hornsby were from the outer fringe of the metropolitan area of Sydney, in rather sparsely populated bushland. The rest have been from country townships or the outlying areas of such townships in the Goulburn Valley in Victoria or in various parts of the Murray River Valley, or rural areas at Canberra, Kangaroo Valley, or Colo Vale, near Mittagong. Bearing this in mind, it is considered that any general conclusions may well apply to southern Australia, but this does not mean that similar results will necessarily be recorded in northern Australia.

(*a*) *Collections from Resting Place Habitats*

In the greater part of our work emphasis has been placed on the collection of fed mosquitoes from their daytime resting places. This has proved easy in some instances where the resting places of particular species have been both known and readily accessible. There are, however, other species of which the resting places are either unknown to us or present serious difficulties to the collector. The difficulties which are known to us are those associated with resting in dense vegetation where the movement of the collector continually stimulates movement of the mosquitoes away from him. In other cases, where resting is presumed to be vegetational on limited circumstantial evidence, other techniques have recently been developed which appear to be relatively successful (see (*b*) below). In our consideration of resting places we exclude the obvious resting of recently emerged individuals in the immediate vicinity of breeding grounds since such situations seldom yield engorged specimens.

(i) *Domestic and Restricted Animal Habitats.*—It will be seen that there is a strong correlation between the result recorded and the most readily available blood source in all domestic and semi-domestic collections. Mosquitoes caught resting inside houses have invariably fed on man, those taken from stables are almost all positive for horse, those from fowl-runs are similarly positive for fowl, and those from dog kennels have all fed on dogs. On the other hand mosquitoes taken from beneath houses are more likely to have fed on animals other than man.

Such an observation is in itself important for it means that we are at liberty to assume that, within the region outlined, similar results are likely to be obtained, and site of collection is a strong indication of the source of the blood meal in those environments wherein there is an obvious and predominating blood

TABLE 1

DETAILED RESULTS OF PRECIPITIN TESTS

Series	Details of Collection	Species	No.	Results	No.	Positive (%)
1	Residence, Hornsby, 8.iv.1952, collected 10 a.m., smeared 10.15 a.m.	*Culex fatigans*	11	+Man	9	
				Negative	2	82
2	Residence, Hornsby, 16.iv.1952, collected 8 a.m., smeared a.m.	*Culex fatigans*	19	+Man	9	
				Negative	10	47
3	Residence, Hornsby, Jan. 1952, collected 9 a.m., smeared 10 a.m.	*Culex fatigans*	12	+Man	12	100
4	Residence, Hornsby, 1.viii.1951	*Aedes alboannulatus*	1	+Man	1	100
5	Fowl house, Hornsby, 8.iv.1952, collected 9 a.m., smeared 9.30 a.m.	*Culex fatigans*	11	+Fowl	9	
				Negative	2	82
6	Fowl house, Hornsby, 16.iv.1952, collected 9 a.m., smeared a.m.	*Culex fatigans*	22	+Fowl	19	
				Negative	3	86
7	Fowl-run, Tuppal, 9.iv.1951, collected, smeared 8-9 a.m.	*Culex fatigans*	7	+Fowl	7	
		Anopheles annulipes	2	Negative	2	78
8	Fowl-run, Tuppal, 10.iv.1951, collected, smeared 8-9 a.m.	*Culex fatigans*	3	+Fowl	2	
				Negative	1	
		Culex annulirostris	1	+Fowl	1	
		Anopheles annulipes	2	+Fowl	1	
				Negative	1	83
9	Fowl-run, Mooroopna, 7.iv.1951. Horses close by, dog next door, man a possibility, ? cat. Collected during afternoon, smeared 2-4 hr later	*Culex fatigans*	159	+Fowl	105	
				+Man	1	
				Negative	53	67
10	Fowl house, Cobram, 5.ii.1952, 1-2 p.m.	*Culex fatigans*	14	+Fowl	14	100
11	Fowl houses, Albury and Wodonga, Dec. 1951 and Jan. 1952. Collected by K. Myers	*Anopheles annulipes*	20	+Fowl	13	
				Negative	7	
		Culex annulirostris	7	+Fowl	4	
				Negative	3	
		Culex fatigans	18	+Fowl	18	
		Culex pipiens australicus	33	+Fowl	31	
				Negative	2	85
12	Horse stable, Cobram, 5.ii.1952. Fowls present, one horse temporarily; 12-1 p.m.	*Culex fatigans*	8	+Fowl	8	100
13	Stable, Mooroopna, 7.iv.1951. Horses only obvious animals, man and dogs in immediate vicinity. Collected afternoon	*Culex fatigans*	34	+Horse	15	
				+Fowl	1	
				Negative	18	48
14	Dog kennels, Cobram, 5.ii.1952, 11-12 a.m.	*Culex fatigans*	11	+Dog	11	100

TABLE 1 (*Continued*)

Series	Details of Collection	Species	No.	Results	No.	Positive (%)
15	Resting and flying around dog and man. Cobram, 2.ii.1952, 8.30-9.0 p.m.	*Aedes theobaldi*	1	+Man	1	
		Aedes vittiger	2	+Man	2	100
16	Septic tank trap, Cobram, 21.ii.1952	*Culex fatigans*	2	+Fowl	1	
				Negative	1	50
17	Small isolated shed, Hornsby gully. Rabbit warren close by, 8.iv.1952, collected 11.30 a.m., smeared 2.00 p.m.	*Culex fatigans* (or possibly another member of this complex)	1	+Rabbit	1	100
18	Haystack, farm, Cobram 29.i.1952	*Anopheles annulipes*	1	+Fowl	1	100
19	From rabbit warrens, various localities in the Albury area, various dates late 1952 and early 1953. Collected by K. Myers	*Anopheles annulipes*	523	+Rabbit	487	
				+Man	1	
				Negative	35	93
20	Taken in trap over exposed rabbit on river flat, Albury, 2.ii.1953. K. Myers	*Culex annulirostris*	97	+Rabbit	96	
				Negative	1	99
21	General shelter collection, dairy, Curlwaa, 22.i.1952. All farm animals and man. Collected 6-7 a.m., smeared 3-4.30 p.m.	*Anopheles annulipes*	45	+Fowl	27	
				+Ox	10	
				+Dog	2	
				+Horse	1	
				Negative	5	
		Culex annulirostris	47	+Fowl	28	
				+Ox	11	
				+Dog	3	
				+Man	2	
				Negative	3	
		Culex fatigans	12	+Fowl	11	
				Negative	1	
		Aedes vittiger	3	+Ox	1	
				+Man	1	
				+Dog	1	
		Aedes alternans	1	+Ox	1	
		Aedes theobaldi	1	+Fowl	1	
		Mansonia linealis	1	+Fowl	1	91
22	House, Wentworth, 22.i.1952. Edge of billabong. From W.C., screen wires, outhouses, etc. Dogs, fowls, ducks, cattle, man, etc. present. Collected 10 a.m., smeared 2.30-3.30 p.m.	*Culex fatigans*	27	+Fowl	21	
				+Man	3	
				+Ox	2	
				+Dog	1	
		Culex annulirostris	4	+Fowl	2	
				+Man	1	
				Negative	1	
		Anopheles annulipes	15	+Ox	10	
				+Man	1	
				Negative	4	91

TABLE 1 (*Continued*)

Series	Details of Collection	Species	No.	Results	No.	Positive (%)
23	Shelters around dairy, West Merbein, 22.i.1952. Cattle, man, fowls, dogs, etc. possible. Early morning collection, smeared 3.0 p.m.	*Anopheles annulipes*	6	+Man	2	
				+Ox	2	
				+Fowl	1	
				Negative	1	83
24	General shelter collection, cow bail, chicken run, wire screens. North Tuppal, 27-31.i.1952	*Anopheles annulipes*	13	+Ox	5	
				+Fowl	3	
				Negative	5	
		Culex annulirostris	7	+Ox	4	
				Negative	3	60
25	Dairy grounds, Curlwaa, 23.i.1952, collected 3.30 p.m., smeared 5.0 p.m.	*Aedes vittiger*	8	+Man	3	
				+Fowl	1	
				Negative	4	
		Aedes theobaldi	1	+Rabbit	1	55
26	River flat, Merbein, 22.i.1952. From stumps. Collected 11 a.m., smeared 2 p.m.	*Culex annulirostris*	1	+Rabbit	1	
		Anopheles annulipes	7	+Rabbit	7	100
27	Sylvan environment, near river bank, Merbein, 21.ii.1952; tree stumps, hollow logs, and in shelter trap. A few cattle and horses, rabbits not obvious owing to earlier myxomatosis epizootic. Collected 9-11.30 a.m., smeared 2-4 p.m.	*Anopheles annulipes*	46	+Rabbit	40	
				Negative	6	
		Culex annulirostris	9	+Rabbit	9	
		Culex pipiens australicus	6	+Rabbit	6	90
28	Tree stumps, river flats, Cobram, 14, 17, 21, and 30.i.1952	*Anopheles annulipes*	55	+Horse	15	
				+Rabbit	4	
				+Man	1	
				+Fowl	1	
				Negative	34	
		Culex annulirostris	10	+Rabbit	2	
				Negative	8	
		Culex pipiens australicus	3	Negative	3	34
29	Kangaroo Valley (largely ground débris and tree stumps for *A. annulipes* and grasses for *C. p. australicus*) Dec. 1952	*Anopheles annulipes*	9	+Rabbit	8	
				Negative	1	
		Culex annulirostris	1	+Rabbit	1	
		Culex pipiens australicus	17	+Rabbit	16	
				Negative	1	92
30	Colo Vale, 17-19.ii.1953 and 9-12.ii.1953. The great majority taken attracted to human bait	*Anopheles annulipes*	1	Negative	1	
		Anopheles stigmaticus	1	Negative	1	
		Aedes vigilax	2	+Man	1	
				Negative	1	
		Aedes notoscriptus	3	+Marsupial	3	
		Aedes alboannulatus	3	+Marsupial	2	
				+Man	1	

TABLE 1 (*Continued*)

Series	Details of Collection	Species	No.	Results	No.	Positive (%)
		Aedes queenslandis	32	+Marsupial	24	
				+Man	5	
				Negative	3	
		Aedes subauridorsum	1	+Man	1	
		Culex pipiens australicus	2	+Rabbit	1	
				Negative	1	84
31	Tidbinbilla, A.C.T., 4.iii.1953 (Mykytowycz)	*Anopheles stigmaticus*	4	+Marsupial	2	
				Negative	2	50
32	Tree boles, Texas, 26.iii.1952 (Dyce)	*Anopheles annulipes*	5	+Rabbit	5	100
33	Rabbit warrens, Moree (Dyce)	*Anopheles annulipes*	7	+Rabbit	7	
		Aedes theobaldi	1	+Rabbit	1	100
34	Canberra, rabbit warrens, 29.i.1953 (Dyce)	*Anopheles annulipes*	12	+Rabbit	11	
				Negative	1	91

source and no great variety of blood sources in the immediate vicinity. It also gives evidence of the range of hosts utilized by particular species of mosquitoes but does not really indicate any degree of preference since the collections in such habitats cannot be considered a random sample to any significant degree. We have simply exploited the opportunities available to us for collecting such samples and have followed a plan of attempting to elucidate obscurities rather than to emphasize what now appears obvious.

Another special environment giving a consistent result has been rabbit warrens. A series of *Anopheles annulipes* collected from such warrens by K. Myers in the Albury area, totalling 523 spots, has given 93 per cent. positive for rabbit, the rest being negative recordings except for one positive for man. The details of these collections in relation to myxomatosis epidemiology will be reported elsewhere (Myers, Marshall, and Fenner, unpublished data).

(ii) *Farmyard Environments.*—Having disposed of these specific man and animal environments, collections have also been made on a number of farms with a considerable variety of blood sources. These collections have been made in and around the various outbuildings, stables, fowl-runs, dairies, etc. and from the exterior of the residence itself but not from inside the residence. As might be expected, these results are more varied both in range of mosquito species and in blood meal sources, and do perhaps give some indication of actual preference although these still require to be regarded with considerable caution.

Table 2 combines the results of three such collections (21, 22, and 23 of Table 1) for the three common species.

From these figures it is obvious that all three species, *Culex fatigans, C. annulirostris,* and *Anopheles annulipes,* feed readily on domestic avian blood sources and at least a degree of preference is shown for such blood by *C. fatigans.* With the other two, *C. annulirostris* and *A. annulipes,* cattle are also freely attacked. There is also a suggestion, but only a suggestion, that *C. annulirostris* is rather more partial to avian blood when it is free to choose between domestic poultry and cattle.

The low figures for horse are due at least in part to the scarcity of these animals. Again, all three species are predominantly night biting, and since man would not commonly be present in the extra-residential environment during the biting period few positives for man could be expected.

Table 2

RESULT OF FARMYARD COLLECTIONS

Species	Total Spots	Fowl	Ox	Dog	Horse	Man	Negative
Culex fatigans	39	32	2	1	—	3	1
C. annulirostris	58	30	15	3	—	3	7
Anopheles annulipes	79	31	27	2	1	3	15

(iii) *River Flat Environments.*—Collections from river flats would be expected to reveal species commonly feeding on rabbits but other blood sources are usually available, e.g. cattle, sheep, horses, and waterfowl of varied species. River flat collections were made specifically in the hope that species feeding on water-birds would be revealed. This hope remained unfulfilled but other interesting information was gleaned.

Table 3

RESULTS OF RIVER FLAT COLLECTIONS

Species	Total Spots	Rabbit	Horse	Fowl	Man	Ox	Negative
Anopheles annulipes	108	51	15	1	1	—	34
Culex annulirostris	20	12	—	—	—	—	8
C. pipiens australicus	9	6	—	—	—	—	3

Series 27 (Table 1) taken from a river flat approximately 2 months after the major myxomatosis epizootic with a seriously depleted rabbit population still gave overwhelming positives for rabbit in the three species collected, *A. annulipes*, *Culex pipiens australicus*, and *C. annulirostris*. This was our first indication of a blood source for *C. pipiens australicus*.

The combined results of the river flat collections (series 26, 27, and 28 of Table 1) are recorded in Table 3.

These results indicate that the three species listed, *A. annulipes, C. annulirostris,* and *C. pipiens australicus*, all find rabbit a very satisfactory blood source.

(iv) *The Hill Stream Environment.*—The vicinity of smaller running streams in hilly or mountainous country provides collecting sites for a variety of species of mosquitoes. Some of these collections cannot be reported here as the common blood source has not yet been revealed, particularly for species such as *Culex pseudomelanoconia*. The blood source possessed nucleated red cells, and, as no reactions occurred with our relatively non-specific anti-fowl serum, we tend to eliminate the possibility of birds. Further, since the common lizard of these environments is *Sphenomorphus quoii*, we suspect this but as yet have no suitable antisera with which to test this suspicion.

However, one such collection is reported in Table 1 (series 29) and it is interesting to note that here again we have strong evidence of rabbit feeding by *A. annulipes* and *C. pipiens australicus.*

(*b*) *Collections from Human Bait*

We have been particularly interested in the blood sources of such species as *Aedes alboannulatus* and *A. queenslandis* but have so far been unsuccessful in locating any significant numbers of engorged resting females. In collections carried out for recovery of myxomatosis virus from wild caught mosquitoes at Colo Vale by D. J. Lee, R. L. Dyce, R. Mykytowycz, and others, it was noticed that a small percentage of the mosquitoes attracted to human bait were already blood fed. These might have been interrupted feeds on nearby collectors, but the specimens were kept for blood smears, and yielded the most interesting information that a considerable proportion actually fed on marsupials (see series 30 of Table 1).

V. Discussion of Results for Individual Species

In assessing the blood preferences, or strictly the frequency of attack on particular blood sources by individual species of mosquitoes, the results of precipitin tests should be linked with other observations, particularly of observed biting of man or other animals under natural conditions.

Under each species listed below an attempt is made to summarize our existing knowledge of their blood-feeding habits. For the collateral field observations we have drawn only on our own observations. When published, the analysis of the various collections made by W. C. Reeves* and others at Mildura in 1951-52 should give far more information on the activity of many of the species dealt with herein.

In Table 4 is recorded an analysis of all precipitin test results for each species. This table does not take into account the other field data of observed biting on man and other animals which is incorporated in the following discussions.

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* This has since appeared: Reeves, W. C., French, E. L., Marks, E. N., and Kent, N. E. (1954).—Murray Valley encephalitis: a survey of suspected mosquito vectors. *Amer. J. Trop. Med. Hyg.* 3: 147-59.

(*a*) *Anopheles annulipes* Walker

There is no question that this species does bite man, but it has also been reasonably obvious that man is not a preferred blood source, and this probably has some bearing on its lack of importance as a vector of malaria (Roberts 1943). That it is widespread in Australia has long been known, and, although it occurs in urban and rural areas, surveys along hundreds of miles of roadways tended to associate its distribution with cattle rather than with man.

TABLE 4

TOTALS FOR INDIVIDUAL SPECIES

Species	Total Tests	Positive for Man	Positive for Fowl	Positive for Rabbit	Positive for Ox	Positive for Dog	Positive for Horse	Positive for Marsupial	Negative Tests
Anopheles annulipes	778	6	47	576	27	2	16		104
A. stigmaticus	5							2	3
Culex fatigans	372	54	197	1*	2	12	15		91
C. pipiens australicus	62		31	24					7
C. annulirostris	184	3	35	109	15	3			19
Aedes alboannulatus	5	2		1				2	
A. queenslandis	32	5						24	3
A. notoscriptus	3							3	
A. subauridorsum	1	1							
A. theobaldi	4	1	1	2					
A. vittiger	13	6	1		1	1			4
A. alternans	1				1				
A. vigilax	2	1							1
A. bancroftianus	1	1							
Mansonia linealis	1		1						
Totals	1464	80	313	713	46	18	31	31	232

*Accurate identification of this specimen was not made at time of capture. It might have been another member of the *fatigans-pipiens* complex.

Field observations have amply demonstrated a decided propensity for cattle biting on the part of *A. annulipes.* At times of low population density of this species it can usually be detected coming to cattle bait during its biting period, and at Cobram, given the choice of man or cow it attacked the cow 15 times more frequently than man. No attempt has been made to substantiate these field observations with special collections for precipitin tests although in general farmyard collections ox was one of the two most favoured blood sources.

The precipitin test results would suggest that rabbit is the most favoured blood source, but there is a definite bias, in that most positives for rabbit came from collections made in rabbit warrens. There is no doubt, however, that rabbits form a major source of the blood supply for *A. annulipes.*

Whether or not a preference exists for either cattle or rabbit is of little practical significance since both are so strongly attacked by this species. One point of interest is that the range of this mosquito is practically Australia-wide, as is the range of cattle, whereas rabbits, though widely distributed, are not available in quite large areas of Australia populated by *A. annulipes.*

Another observation of significance is that *A. annulipes* is very frequently found in small numbers resting in fowl sheds. That poultry are a blood source is evidenced by the precipitin tests where in the mixed farmyard environment biting of poultry and cattle is about equal.

Horses are also attacked and it is interesting that collections from typical river flat resting places, exclusive of rabbit warrens, still show a marked predilection for rabbit feeding but where horses have also been present a reasonable recovery of horse-fed specimens has been made.

In conclusion one would presume cattle and rabbits to be the two major sources of blood for *A. annulipes* but where poultry and horses are present within the feeding range of this species they are attacked, but to a lesser degree. Dogs and man are also attacked but to a very significantly lesser degree.

It is also worthy of comment that in a species which we must consider as an indigenous one in Australia its major sources of blood are from animals which have been introduced to the country since the time of white settlement. Whatever trend of speculation this may lead one to, at least there is a strong suggestion that the population level of this species is nowadays considerably greater than it was 150 years ago.

Excluding the close relationship of *A. annulipes* to rabbit warrens, at least in inland areas, recoveries of fed adults have been made in a variety of different places. In the semi-domestic environments, outhouses, animal sheds, and fowl-runs have all been useful. Privies, particularly when they are decidedly odorous, have also been a fairly reliable source. In the sylvan environment cavities in tree stumps, beneath logs, in ground cavities, and débris on the ground have yielded good returns. Other recoveries have been from cavities in rocky creek banks, in grasses and sedges, and under bridges.

(*b*) *Anopheles stigmaticus* Skuse

This species has been used in malaria transmission experiments by Mackerras and Roberts (1947). They recorded that it bit very reluctantly in the laboratory and that much time and patience were needed to induce even a few individuals to engorge on man. We are aware of only three records of this species biting man in the field and we have not observed it attacking any other animals. Obviously man is not a usual source of blood for this species, and, although our precipitin tests record two positives for marsupial blood, we suspect that our range of antisera is not sufficiently wide to reveal other possible blood sources.

Although by no means a common species, engorged females have been collected in rock crevices close to the water in rocky creeks in hilly country, and occasionally in ground débris.

(*c*) *Culex fatigans* Wiedemann

For an adequate understanding of this species we must emphasize its exceptionally strong tendency to bite during the night in houses or other shelters. It is undoubtedly a strongly anthropophilic species and all collections from within houses have been man-fed. Again, as the precipitin tests reveal, it has an equally strong habit of attacking poultry and it is probably even more abundant in suitable poultry sheds than it is in human habitations. Where horses are stabled overnight it will also attack these with reasonable freedom. Again, where dogs are suitably kennelled, dog-fed specimens are recovered. Cattle are not significantly attacked by this species (but we must admit that no collections have been made where cattle have been under a roof overnight) and rabbits apparently not at all.

This is undoubtedly the easiest of all species to collect in the engorged condition since it rests after its blood meal in the shelter wherein biting took place. It can be recovered inside houses, particularly in darker corners and cupboards, but also generally on walls and ceilings in the early part of the morning, and in sheds, outhouses, and especially fowl sheds and stables.

We have seldom observed *Culex fatigans* biting out of doors, or rather outside the confines of some sort of artificial shelter whether it be house, stable, lean-to fowl shed, or motor car. Such shelters are used for resting after feeding but some apparently do not provide ideal conditions throughout the day, especially where sunshine has access to the inside of the shelter. In such cases movement takes place some time after feeding to more suitable diurnal resting places. Many unengorged specimens will also be recovered from suitable diurnal resting places.

We conclude from this that *Culex fatigans*, although having a strong association with domestic birds (sparrows could also be included with these because of the association of *C. fatigans* with the transmission of bird malaria), there is little evidence to suggest that the species comes into contact with native birds and in particular the various species of waterfowl.

(*d*) *Culex pipiens australicus* Dobrotworsky and Drummond

C. pipiens australicus has only recently been differentiated as a distinct entity of the *fatigans* complex although for quite a few years it has been recognized as the sylvan counterpart of the domestic *C. fatigans* (see Dobrotworsky and Drummond 1953).

Until quite recently we had no knowledge of its blood sources, but the present precipitin tests reveal it to be both a rabbit- and fowl-biting form. For the time being emphasis should be placed on rabbit as a blood source since this subspecies is far more commonly taken in sylvan environments than in semi-domestic ones. Nevertheless further study is desirable as it is still possible that other blood sources will be revealed. Although we have recorded it attacking man on three occasions this subspecies is in no sense anthropophilic, nor does it appear to commonly attack the usual range of farmyard animals.

C. pipiens australicus has been recovered as engorged specimens quite rarely in farmyard shelters except perhaps the Albury fowl-run collections of

K. Myers. Resting in tree stumps and similar situations on river flats has been the most successful situation so far exploited. Grasses and sedges have been next but fewer fed specimens are recovered. Ground débris and ground cavities have also yielded small numbers.

(*e*) *Culex annulirostris* Skuse

The precipitin tests reveal that this species has a strong tendency to attack rabbits. They also reveal that poultry and cattle are also attacked with some frequency, but man rather less often. To interpret these results, it is necessary to record that the biting of this species is largely, but not consistently, in the early hours of darkness and usually in the out-of-door and especially the sylvan environment. It does not ignore man but attacks cattle with greater frequency, a ratio of 5 : 1 cattle to man being recorded at Cobram. In the farmyard environment, when this is close to its semi-permanent river breeding grounds, poultry are most frequently attacked, but cattle, dogs, and man are also recorded as blood sources. Field observations have recorded it as attacking cattle, man, rabbits, and cats.

Cattle and rabbits are probably the predominant blood sources, fowls are important in certain environments, and man is rather more frequently attacked than the precipitin test data suggest. Our field evidence would suggest that *C. annulirostris* is a little more anthropophilic than *A. annulipes.*

C. annulirostris is often an obvious component of the farmyard collections, but is not usually taken within dwellings. Most recoveries of engorged specimens have come from tree stumps on river flats; occasionally it has been taken from grasses and sedges and in ground débris.

(*f*) *Aedes alboannulatus* (Macquart)

Most specimens of this species are taken actually biting man when man enters its environment, especially bushy gullies. We have suspected that man is not an important blood source for this species in nature and it is most interesting to record the two tests positive for marsupial blood (Table 4).

This species very occasionally enters houses when these are in close proximity to bush, and fed adults have been recovered on one or two occasions. Otherwise we have failed to collect engorged specimens in field resting places although occasional blood-fed specimens are taken attracted to man.

One precipitin test for this species, forwarded by A. L. Dyce from Moree (not recorded in the foregoing tabulations) gave a double positive reaction to man and rabbit. This and one other are the only definite records we have of this species feeding on rabbits as revealed by precipitin tests. Double feeds have been rare in our experience but it does agree with our observations elsewhere that engorged females may still attempt to bite a second time, and are still responsive to a blood source such as man coming within their range of detection even when at least partially engorged.

This species has also been observed attacking a cow and a dog but both of these are single observations. Recently, in trapping experiments, we have observed this species attacking caged rabbits in the field.

(*g*) *Aedes queenslandis* (Strickland)

This species is similar in most respects to *A. alboannulatus*. It occupies roughly the same area of distribution, its breeding habitats are similar, and it is closely related morphologically.

It is also taken most frequently attacking man but again we have suspected that man is not its normal blood source. From our collections of this species attracted to man as bait we have quite strong evidence that it is predominantly a marsupial-feeding species. The strength of the observed reactions in our precipitin tests with the non-specific anti-possum serum and the habitat in which the specimens were collected would suggest, but by no means conclusively, that the actual blood source would be possums or phalangers. Rabbits are also attacked by this species as we have observed on caged rabbits exposed in the field.

Again we failed to discover characteristic resting places for engorged individuals of this species.

(*h*) *Aedes notoscriptus* (Skuse)

This species is well known to be anthropophilic in the domestic environment. It is interesting to record that we have also found it to be a marsupial feeder. Our field observations have also disclosed it biting dogs and cattle.

In the data before us we have no significant records of the resting places of this species. Resting inside houses after feeding does occur but is in no way comparable to the feeding activity.

(*i*) *Aedes subauridorsum* Marks

An uncommon species, *A. subauridorsum* is very occasionally taken attacking man in the bush. The one positive precipitin test recorded for this species was apparently one taken immediately after feeding on man. No resting places are known for this species.

(*j*) *Aedes theobaldi* (Taylor)

Although only a total of four fed specimens have been taken for precipitin tests on this species, giving positives for man, fowl, and rabbit, many field observations of its biting have been made.

A. theobaldi is predominantly a day-biting mosquito although in certain areas climatic circumstances tend to divert peak biting to the early evening. It attacks obvious blood sources such as man, cattle, horses, sheep, and dogs with equal avidity but although it attempts to bite poultry it is seldom successful in daylight. Cats are also attacked. In our Cobram records where significant differences were recorded for biting rates of other species as between cattle and man, there was little evidence of cattle being more attractive than man to *A. theobaldi*.

This species obviously lacks discrimination in its blood-feeding but we do not know whether it attacks birds to any significant degree.

Recently emerged specimens fly up from grasses to attack animals or man, others at times appear to descend from low trees. Blood-fed adults have seldom been taken and we have been unable to disclose any characteristic resting places of such individuals.

(*k*) *Aedes vittiger* (Skuse)

Attacks on man by this species are well known and in many respects its behaviour is similar to that of *A. theobaldi.* It frequently rises from rank grass to attack man and other animals during the day but may also be very active after dark. It readily follows man into motor vehicles and often travels long distances in this way.

Our precipitin tests reveal that it has been recorded biting man, ox, dog, and fowl and our field observations have shown it attacking horse, sheep, and cat as well. It attacks principally in the sylvan environment and is rarely taken resting in shelters except when these are close to its breeding grounds. Recovery of fed adults in the field has not been satisfactory, most specimens taken having recently attacked the collector. Again we have no knowledge of the extent to which birds are attacked by this species.

(*l*) *Aedes alternans* (Westwood)

Only one precipitin test has been done for this species, giving a positive for ox. Nevertheless *A. alternans* is widely known as a pest of man, cattle, and horses. It attacks viciously in the vicinity of its breeding grounds during the day and in the early evening bites in the open and occasionally on open verandahs. Whether or not birds are attacked is unknown.

(*m*) *Aedes vigilax* (Skuse)

Again a pest of man, cattle, and horses wherever it is abundant and a vicious day and evening biter, it would seem likely that with both *A. vigilax* and *A. alternans* some other blood source is utilized. The possibility of the estuarine bird fauna exists but we have no evidence one way or another on this point. The single positive precipitin test for man is recorded for the sake of completeness of the Colo Vale records.

(*n*) *Mansonia linealis* (Skuse)

Until recently little has been known about this species other than that it does attack man from time to time. Our single positive precipitin test reveals that it will attack fowls and at Kangaroo Valley it was observed attacking man and dog with approximately equal avidity.

VI. Epidemiological Conclusions Related to Biting Activity

The use of precipitin tests on the stomach contents of engorged mosquitoes has certain limitations. Firstly, one does not always have as wide a range of antisera as one would desire, and secondly, some species of mosquitoes are so elusive that it is difficult to obtain satisfactory material for precipitin testing.

Nevertheless, when precipitin tests are used in conjunction with observations of biting in the field, a useful body of information is amassed. The precipitin tests themselves are most useful in proving what is otherwise suspected, in arriving at an appreciation of the range of hosts favoured by night-biting mosquitoes, and in giving clues to the unsuspected biting activity of species whose habits are otherwise imperfectly known.

To use precipitin tests as a tool in the elucidation of mosquito blood-feeding requires deliberate collecting of particular species in particular environments. In this we consider we have had some measure of success, but there are some species and some environments which so far we have not had suitable opportunities to investigate.

In particular, we have not been able to gather any useful information on the mosquitoes attacking wild birds, and especially water-birds. In this connection, we would like to record that *Culex globocoxitus,* a common, perhaps dominant, riverine species of the Murray Valley in South Australia, inhabits an environment shared equally by many waterfowl. We consider it highly probable that *C. globocoxitus* is ornithophilous, an opinion also expressed by Dobrotworsky (1953).

Another species which may have some association with birds in permanent swamp or riverine habitats is *Mansonia linealis,* which we have occasionally taken resting in willow trees on river margins.

Apart from these, the species which we have been able to collect in riverine environments, namely *Anopheles annulipes, Culex annulirostris,* and *C. pipiens australicus,* have yielded no evidence (with the exception of a single avian positive for *A. annulipes*) that birds are utilized as a blood source in this type of environment, despite the fact that all three attack domestic poultry. Precipitin testing of engorged individuals from typical waterfowl environments is required to establish whether these species do feed on such birds.

If, as seems more than probable, mosquitoes do act as vectors of the virus of Murray Valley encephalitis from bird to bird, then any of the species mentioned above might be implicated. If *C. globocoxitus* were disclosed as a vector, then the virus would have, as far as we now know, little if any contact with man and the animals or birds in his domestic environment. With the others, however, contact with the domestic environment could be established from time to time, particularly with *A. annulipes* and *C. annulirostris,* and to a considerably lesser extent, because of its more patchy distribution, *M. linealis.* Again, neither the latter nor *C. pipiens australicus* can be considered characteristic members of the farmyard or semi-rural environment, in so far as our observations have disclosed, despite the common occurrence of breeding of *C. p. australicus* in semi-rural areas, whereas *A. annulipes* and *C. annulirostris* are commonly and widely found under such circumstances. However, since McLean (1953) has shown that *A. annulipes* is not hospitable to Murray Valley encephalitis virus this species can be ignored.

The elucidation of the bird-biting complex of mosquitoes is of importance both for an understanding of the full cycle of Murray Valley encephalitis and of the transmission of various fowl pox viruses. If we can disclose the species commonly attacking birds, then selective collections for virus recovery can be made when it is suspected or known that one or other of these viruses is active.

In both cases a basic working hypothesis is that bird-to-bird transmission occurs through the agency of those mosquitoes most frequently attacking birds and that from time to time a break-through to domestic birds is occasioned by mosquitoes having opportunity to attack both wild and domestic birds. Then, since it has been shown to be capable of transmitting the virus (McLean 1953), there is no doubt that *Culex fatigans* must be the primary suspect for transmission of Murray Valley encephalitis amongst domestic poultry, and a strong suspect for the transmission of fowl pox. In Murray Valley encephalitis it must be equally the primary suspect for transmission to man.

Especially in Murray Valley encephalitis such a conclusion is not fully justified on the evidence of biting habits alone. We must also take into account certain other types of evidence, as follows:

(*a*) McLean (1953) has shown 11 species of mosquitoes to be hospitable to M.V.E. virus. These are *Culex annulirostris, C. fatigans, C. globocoxitus, C. pipiens australicus, C. pipiens molestus, Aedes camptorhynchus, A. queenslandis* (as *A. occidentalis*), *A. notoscriptus, A. theobaldi, A. vigilax,* and *A. vittiger.* Of these at least *C. annulirostris, C. fatigans, A. queenslandis,* and *A. vigilax* are also capable of transmitting the virus to chickens (McLean 1953). This is strong evidence that there is not a high degree of specificity established between virus and mosquito, at least within a wide range of culicine species. This is closely parallel to the findings for Japanese *B* encephalitis for which Reeves and Hammond (1946) found four species of *Culex*, four of *Aedes*, and two of *Culiseta* to be successful laboratory vectors. Hence the general belief that a complex of vectors is involved in the transmission of M.V.E. is not unjustified on existing evidence.

(*b*) The observations of Anderson *et al.* (1952) and Miles and Howes (1953), which both arrive at a 20 per cent. infection rate among humans in the Murray Valley, indicate a very high degree of transmission to man when one considers that the period of viraemia in birds is likely to be short and non-recurring. In fowls a period of 3 days is given by Anderson *et al.* (1952) and up to 8 days for the silver gull (*Larus novohollandiae*) by Miles (1952). Such figures lead us to believe that a 20 per cent. infection rate in man is high and can only be occasioned by a species of mosquito in close contact with domestic poultry and man rather than by species casually fulfilling this association.

C. fatigans then is undoubtedly the primary suspect, and failing the disclosure of other pertinent information in the meantime, it must be the species against which active measures should be taken in order to break the cycle of transmission to man in future outbreaks.

A further observation of interest is the even higher percentage of humans showing antibody to Murray Valley encephalitis recorded for the Northern Territory, the figure recorded by Miles and Howes (1953) being 26 per cent. It seems most unlikely that the same cycle of transmission to man would obtain in the Northern Territory aborigines as in whites in the Murray Valley area.

A final point to note is the high percentage of infection in horses within the outbreak area. A figure of 50 per cent. or more is given by both Anderson *et al.* (1952) and Miles and Howes (1953). It would appear that horses are attractive to mosquitoes likely to have fed on other hosts for the virus and hence in the choice of animal baits for collecting mosquitoes for virus recovery during the course of an outbreak, horses should perhaps be given preference over cattle.

The view expressed above, that *C. fatigans* has merited first consideration as the vector route of Murray Valley encephalitis to man, is perhaps contrary to the views expressed by Burnet (1952) and Anderson *et al.* (1952), who place more emphasis on the role likely to be played by *C. annulirostris.* This change of emphasis is due to the well-established fact that man is extensively attacked by *C. fatigans* on the one hand and that on the other we have little evidence to support a view that man is sufficiently frequently attacked by *C. annulirostris* to produce an infection rate approaching 20 per cent. Nevertheless, further study of the biting activity of *C. annulirostris* is highly desirable since important differences in possible control measures are involved.

With fowl pox so little is known of the incidence of the disease and its occurrences in birds other than fowls that there are few data on which to base speculation. An obvious possibility for fowl-to-fowl transmission is *C. fatigans* but the apparent seasonal limitation of the disease to autumn in the Sydney area would perhaps tend to implicate *A. annulipes.* Such a suggestion is contingent on the seasonal incidence being related to vector activity which in any case is a very precarious assumption, since the determining factor might just as readily be the availability of susceptible birds in late summer and autumn.

However, a study of fowl pox virus and the means by which it comes into contact with poultry may lead to observations of considerable interest in the understanding of the avian cycle of Murray Valley encephalitis.

An entirely different problem is involved in the transmission of myxomatosis. Here we are only concerned with the movement of the virus from one rabbit to another, and, since the transmission of the disease is mechanical (Fenner, Day, and Woodroofe 1952), any mosquitoes likely to bite rabbits twice are highly presumptive vectors. The evidence presented strongly implicates *A. annulipes* and also *C. annulirostris* and *C. p. australicus.*

We have already mentioned the close association of *A. annulipes* with cattle, and there is a possibility that transmission of ephemeral fever of cattle might be more closely linked with this species than was earlier suspected (Mackerras and Mackerras 1940). Nevertheless, the previous guess that sandflies (Ceratopogonidae) might be the transmitting agent still remains a distinct possibility.

Finally, the demonstration of *A. annulipes* as having a wide range of blood sources, with particular preference, where available, for cattle and rabbits, does confirm the opinion of most workers as to why it so seldom acts as a vector of human malaria despite its being a satisfactory experimental one (Roberts 1943).

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REGENERATION AND THE MOULTING CYCLE IN *BLATTELLA GERMANICA* L.

II. SIMULTANEOUS REGENERATION OF BOTH METATHORACIC LEGS

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Summary

At 29°C, 70 per cent. relative humidity, simultaneous removal of both metathoracic legs at the proximal autotomy plane in first instar *Blattella germanica* results in the appearance at the first moult of either a symmetrical pair of differentiated regenerates or a symmetrical pair of undifferentiated papillae, but not of intermediate stages in differentiation. Papillae at the first moult are followed by differentiated regenerates at the second. Simultaneously produced regenerates are not distinguishable from those arising from unilateral operations.

The first moult of simultaneously regenerating animals is not delayed when a pair of papillae is produced, but a delay somewhat greater than the age of the animal at operation is associated with appearance of a pair of regenerates at the first moult. The second moult of simultaneously regenerating animals producing papillae at the first is delayed by about one day. Comparison with unilaterally regenerating animals shows a slightly but significantly increased delay in moulting related to simultaneous regeneration. This is probably due to increased demands for metabolites in producing two regenerates, added to the disturbance of endocrine balance already postulated from experiments on unilateral regeneration.

I. Introduction

Regeneration of one metathoracic leg during the first instar in *Blattella germanica* has been shown to follow an "all or nothing" principle, related to the existence of a "critical period" during the instar. Removal of the leg before this period leads to the appearance at the first moult of a highly differentiated regenerated leg with a tetramerous tarsus. Loss of the leg apparently subjects the instar to a fresh start, so that the moult is delayed by a period roughly equal to the age of the animal at the time of removal of the leg. No such delay results from removal of the leg after the "critical period," when an undifferentiated papilla appears at the first moult, and a regenerate not until the second.

In reporting these results, O'Farrell and Stock (1953) tentatively interpreted them in terms of a postulated interaction between regeneration and the endocrine balance involved in moulting. An alternative hypothesis discussed was that competition between regenerative and normal growth for metabolites other than hormones (e.g. structural or energy-producing materials) might be responsible for the observed delay in moulting, but this was considered less satisfactory.

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Assuming that both metathoracic legs can be regenerated simultaneously, further evidence should be obtainable by simultaneous removal of both of them, which would double the quantity of tissue to be regenerated. The present paper describes experimental work along these lines.

II. Material and Methods

The experimental methods used in this work were substantially those described by O'Farrell and Stock (1953). Since it was thought necessary to work on large numbers of animals under one set of physical conditions such that the first instar of controls would be completed in about 6 days from hatching, all cultures and experimental animals were kept in a constant-temperature room at 29°C, 70 per cent. relative humidity (R.H.) (± 0·5°C and approx. 8 per cent. R.H.).

Simultaneous removal of both metathoracic legs at the proximal autotomy plane was carried out on each of the first 4 days of post-embryonic life, using a total of about 1000 experimental animals and 1100 controls. A further 200 animals subjected to removal of only one metathoracic leg provided a direct comparison between the effects of unilateral and simultaneous operation in the same material and under the same conditions.

III. Results

Locomotion, although modified, was not found to be apparently impaired by the absence of both metathoracic legs, at least during the first and second instars, the period covered by these experiments. It appeared that simultaneous regeneration of both metathoracic legs took place during the first instar in much the same way as the regeneration of one. Operations late in the instar gave rise to a symmetrical pair of undifferentiated papillae, appearing at the first moult (Plate 1, Fig. 1), while those early in the instar led to the appearance at the first moult of a symmetrical pair of well-differentiated regenerates with tetramerous tarsi (Plate 1, Fig. 2). In only two instances did any structure suggesting an intermediate condition between papilla and regenerate appear externally at the moult, taking the form on both occasions of a pair of rather elongate and irregularly shaped papillae with what may have been signs of incipient segmentation. Such rare exceptions to the "all or nothing" principle were regarded as probably due to accidental injury and did not seem to warrant the suggestion that the principle is not operative in simultaneous regeneration.

Variations between individual animals in the size and minor morphological detail of simultaneously produced regenerates were observed, but did not seem to be consistently related to the timing of the operation or the moult. Further, no consistent morphological or size difference was noticed between the simultaneously produced regenerates and those resulting from unilateral operation, an example of which is shown for comparison in Plate 1, Figure 3. Similar general statements apply to the symmetrical pair of highly differentiated regenerates appearing at the second moult (Plate 1, Fig. 4), following production of a symmetrical pair of undifferentiated papillae at the first moult. Symmetry in size and differentiation appeared to be characteristic of all simultaneously produced pairs of regenerates or papillae.

TABLE 1

FIRST MOULT OF ANIMALS PRODUCING REGENERATES AFTER SIMULTANEOUS REMOVAL OF BOTH METATHORACIC LEGS, COMPARED WITH THAT OF THE CORRESPONDING CONTROLS AND OF UNILATERALLY REGENERATING ANIMALS

Age at Operation (days)	Mean Age (days) and Numbers Surviving at First Moult of		Mean Age Difference (days) at First Moult Between	
	Controls	Animals Undergoing Simultaneous Operation	Controls and Simultaneously Regenerating Animals*	Simultaneously and Unilaterally Regenerating Animals*
0-1	6·38±0·06 (114)	7·58±0·05 (77)	1·20±0·08	0·04±0·12 (27, 39)
1-2	6·37±0·04 (348)	8·26±0·03 (339)	1·89±0·05	0·29±0·07 (110, 103)
2-3	6·30±0·04 (415)	9·03±0·04 (331)	2·73±0·05	0·64±0·11 (82, 55)
3-4	6·48±0·11 (51)	10·52±0·12 (27)	4·04±0·16	No data

*Computed by the method of Simpson and Roe (1939, pp. 192-3).

The time relations observed between unilateral and simultaneous operation, regeneration, and moulting are summarized in Tables 1 and 2. The appearance of regenerates at the first moult, whether resulting from unilateral or from simultaneous operation, was associated with a delay in the moult approximating the age of the animal at operation (Table 1). Operations performed on the first day of post-embryonic life, however, produced a delay in the moult exceeding 1 day; this recalls the similar results reported for unilateral regeneration by O'Farrell and Stock (1953). On the fourth day of post-embryonic life, operations resulted in the appearance at the first moult (which was not delayed) of papillae in the great majority of animals (Table 2); only a few exceptionally slow-growing batches produced regenerates at a delayed first moult (Table 1). Similarly, the majority of batches undergoing operation on the third day of post-embryonic life produced regenerates at a delayed first moult (Table 1), and papillae appeared at an undelayed first moult only in exceptionally fast-growing material (Table 2).

It is clear from Table 1 that there was a considerable similarity between the moulting of unilaterally and simultaneously regenerating animals, apparently amounting to identity for those undergoing operation on the first day of post-embryonic life. Operations performed on the second and third days of post-embryonic life, however, gave rise to the apparently highly significant differences between singly and simultaneously regenerating animals shown in Table 1, column 5, where figures in parentheses show, for each day of operation, the respective numbers of simultaneously and unilaterally regenerating animals which were available for the purposes of this comparison.

No direct comparison between singly and simultaneously regenerating animals producing papillae at the first moult appeared to be necessary. Table 2 shows that the first moult in animals producing papillae, after undergoing simultaneous operations on either the third or fourth day of post-embryonic life, was not delayed in comparison with that of the corresponding controls. Their second moult, at which regenerates appeared, was significantly delayed by comparison with that of the controls (Table 2, column 6).

Table 2

FIRST AND SECOND MOULTS OF ANIMALS PRODUCING PAPILLAE AT THE FIRST AND REGENERATES AT THE SECOND MOULT, AFTER SIMULTANEOUS REMOVAL OF BOTH METATHORACIC LEGS, COMPARED WITH THOSE OF THE CORRESPONDING CONTROLS

Age at Operation (days)	Mean Age (days) and Numbers Surviving				Mean Difference (days) at Second Moult, Between Controls and Experimental Animals*
	At First Moult		At Second Moult		
	Controls	Experimental Animals	Controls	Experimental Animals	
2-3	5·85±0·05 (122)	5·76±0·06 (60)	11·56±0·09 (102)	12·41±0·14 (45)	0·85±0·17
3-4	6·29±0·06 (137)	6·09±0·05 (128)	11·47±0·16 (78)	12·57±0·08 (81)	1·10±0·13

*Computed by the method of Simpson and Roe (1939, pp. 192-3).

IV. Discussion

The results here described demonstrate the possibility of regeneration, within one instar, of both metathoracic legs after their simultaneous removal at the proximal autotomy plane. Such simultaneous regeneration conforms to the general pattern of the "all or nothing" principle and associated "critical period," already described for unilateral regenerations by O'Farrell and Stock (1953). Further, the regenerates produced do not differ in any obvious respect from those arising from unilateral operations, and the members of a pair are symmetrical with respect to one another, so that they conform *as a pair* to the "all or nothing" principle. Hence there seems to be some justification for concluding that the pair of regenerates appearing after a simultaneous operation represent approximately twice as much expenditure of structural material and metabolic energy as would be required by regeneration of a single member of the pair at the same time in the instar.

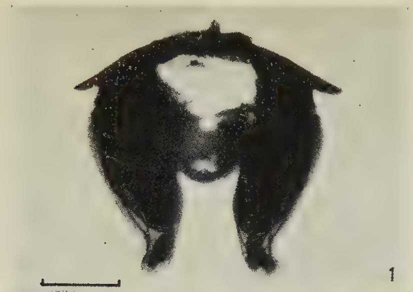
If this conclusion be accepted, and it is postulated that the delay in the first ecdysis imposed by production of a regenerate is primarily due to the draining off by regeneration of certain quantities of material otherwise available for general growth, then simultaneous regeneration might be expected to produce something approaching twice as great a delay in moulting as results from unilateral regeneration. In fact, as Table 1 shows, simultaneous regeneration produces a significantly greater delay in moulting than unilateral regeneration, but the magnitude of this difference is small in comparison with the postponement of moulting, in both cases, by a period approximating the age of the animal at operation. In short, the increased delay in moulting resulting from simultaneous regeneration is not proportionate to the doubling of the quantity of tissue to be produced. Such a proportion, however, may possibly be realized in the delayed second moult of animals producing papillae at the first moult. This delay is significant for simultaneously regenerating animals (Table 2, column 6), but was not found to be statistically confirmable in singly regenerating material by O'Farrell and Stock (1953).

Except for operations performed on the first day of post-embryonic life then, the present work suggests that simultaneous regeneration always results in a slight but definite increase in the delay in moulting observed for unilateral regeneration. Increased blood loss and/or a more profound wound shock resulting from the loss of two legs instead of one may possibly be responsible for this. However, this interpretation is difficult to justify for the results of operations performed after the critical period (when the delay in moulting is transferred to the next instar) or on the first day of post-embryonic life (when both singly and simultaneously regenerating animals show an identical but unexpectedly prolonged delay in moulting). Moreover, the data as a whole appear rather more internally consistent than would be expected if the time of moulting were being seriously affected in experimental animals, but not controls, by uncontrolled variables such as blood loss or wound shock.

On the whole, the present results appear to support an interpretation in which a major part is played by disturbance of the endocrine balance on the lines already suggested by O'Farrell and Stock (1953). A minor part, represented by the slightly increased delay in moulting of simultaneously regenerating animals, may be ascribed either to blood loss and wound shock, or, perhaps more plausibly, to the effects of successful competition by regeneration for the available limited supplies of structural or energy-producing materials, or both.

V. Acknowledgments

The authors are indebted to the Research Committee of the New England University College (now the University of New England) for financial assistance in the purchase of equipment.



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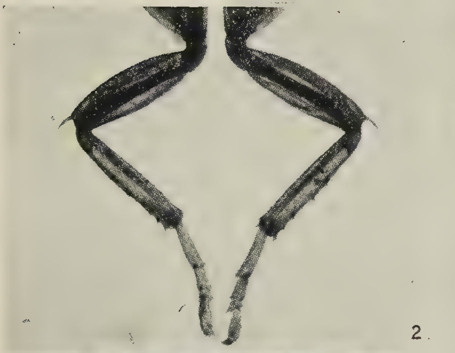

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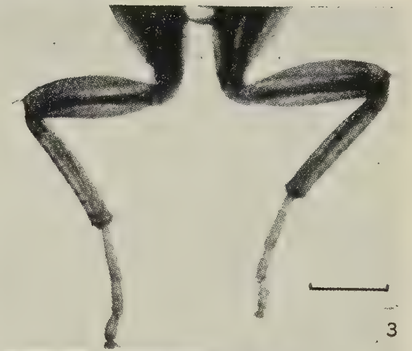

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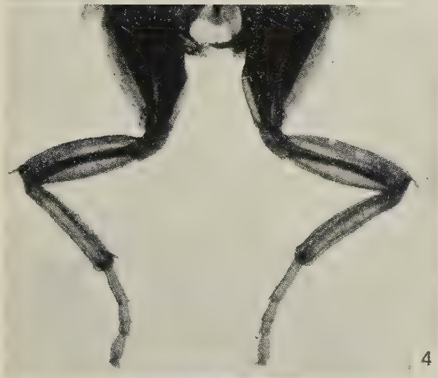

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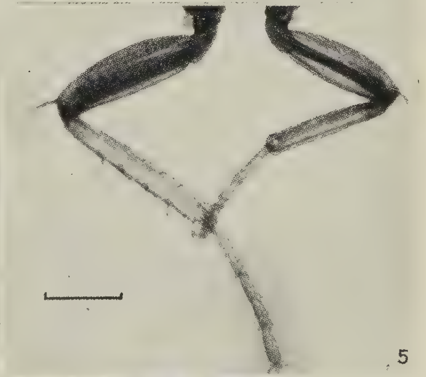

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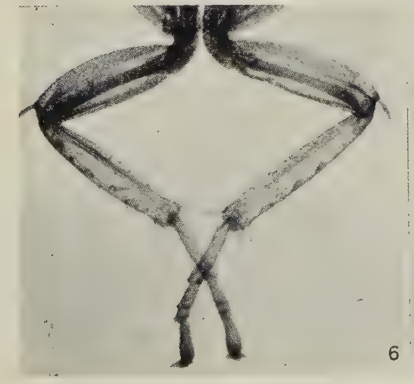

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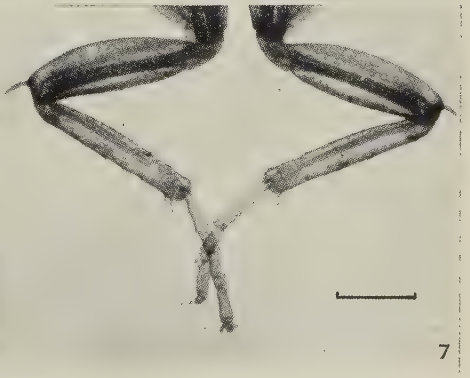

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EXPLANATION OF PLATE 1

Blattella germanica L.

All preparations fixed in alcoholic Bouin, stained in alcoholic light green, and photographed from the anteroventral aspect. Scales indicate 0·5 mm.

Fig. 1.—Symmetrical pair of smoothly rounded papillae appearing at the first moult after simultaneous removal of both metathoracic legs on the fourth day of post-embryonic life. Fixed on the fourth day of the second instar to show advanced differentiation within the old coxae, and extending into the papillae, of the regenerates due to appear externally at the second moult.

Figs. 2, 3.—Symmetrical pairs of regenerates appearing at the first moult after simultaneous removal of both metathoracic legs (Fig. 2) on the second, and (Fig. 3) on the fourth day of post-embryonic life.

Figs. 4, 5.—Regenerates appearing at the first moult after operations performed on the third day of post-embryonic life, (Fig. 4) after simultaneous removal of both metathoracic legs; (Fig. 5) after removal of the left metathoracic leg only.

Figs. 6, 7.—Symmetrical pairs of regenerates appearing at the second moult, following the appearance of papillae at the first moult, in animals subjected to simultaneous removal of both metathoracic legs on (Fig. 6) the third, and (Fig. 7) the fourth day of post-embryonic life.

A STUDY OF CYSTEINE DESULPHYDRASE IN CERTAIN INSECTS

By R. F. Powning*

[*Manuscript received February* 11, 1954]

Summary

An enzyme capable of liberating H_2S from *l*-cysteine *in vitro* (cysteine desulphydrase) is present in insect tissues. The clothes moth (*Tineola bisselliella* Humm.) has a highly active desulphydrase apparently concentrated in the gut, whilst some other insects have little or no activity. Aqueous enzyme solutions from insects are unstable, their optimum pH ranges between pH 7·8 and 8·9, they are inhibited by NaCl and KNO_3 (both at 0·42M), and As_2O_3, $NaHSO_3$, and phenylhydrazine (all at 10^{-2}M), but they are not affected by semicarbazide, thiourea, sodium pyrophosphate, or sodium fluoride (all at 10^{-2}M). KCN (10^{-2}M) activates clothes moth preparations, has no effect on those of carpet beetle, and inhibits some other insect enzymes.

Cystine, methionine, homocysteine, glutathione, or thioglycollate do not function as substrates for *Tineola* desulphydrase. The Michaelis constant for *Tineola* desulphydrase under the conditions used is $3 \cdot 8 \times 10^{-2}$M. Dialysis of the clothes moth enzyme against water results in a considerable loss of activity but this did not occur to the same extent in buffered solutions. Activity cannot be restored by the addition of Mn, Zn, or Mg ions (2×10^{-2}M), vitamin B_6 derivatives, folic acid, or boiled undialysed enzyme.

I. Introduction

It has been shown by Waterhouse (1952*a*) that larvae of the clothes moth (*Tineola bisselliella* Humm.) convert heavy metals, ingested with the food, to insoluble sulphides and excrete them in the faeces. These larvae also have a low oxidation-reduction potential in their midgut which is thought to cause the initial stages of digestion of wool (Linderstrøm-Lang and Duspiva 1936). The presence of a reducing substance such as hydrogen sulphide in the *Tineola* gut is therefore of importance in studying the mechanism of wool digestion and metal detoxification by these insects.

The production of H_2S from certain sulphur compounds by enzymes, the desulphydrases, has been observed in bacteria and mammalian tissues (Desnuelle 1939; Fromageot, Wookey, and Chaix 1941; Smythe 1942; Azarkh and Gladkova 1952) and, in view of the presence of cystine in the excreta of clothes moth and carpet beetle larvae (Powning 1953), these and other insects have been examined for desulphydrase activity. Some properties of such an enzyme from *Tineola* larvae have been examined and compared with those of other enzymes from other sources.

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CORRIGENDUM

Page 309, Fig. 1: The apparatus on the right is that referred to in the legend. The remainder of the figure should be ignored.

II. Methods and Materials

Cysteine desulphydrase activity was measured by estimating H_2S production from *l*-cysteine (L. Light and Co.) in Thunberg tubes which had been modified as shown in Figure 1. One arm of the apparatus contained 2·4 ml of the enzyme-substrate mixture (see text below for composition), the other

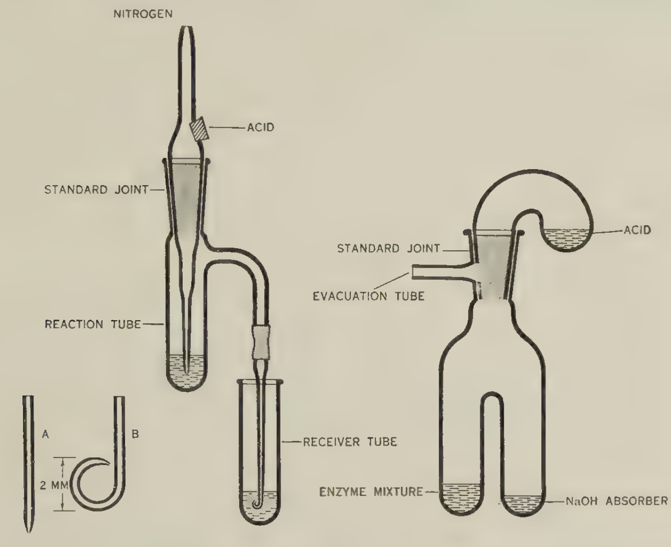

Fig. 1.—Apparatus for estimation of cysteine desulphydrase activity by vacuum diffusion of the hydrogen sulphide produced.

0·5 ml 0·5N NaOH; the stopper contained 0·5 ml 10 per cent. H_2SO_4. The freshly prepared solutions of cysteine and other reagents were adjusted to the desired pH and stored temporarily under anaerobic conditions in Thunberg tubes. The prepared modified Thunberg tubes were evacuated, filled with O_2-free nitrogen, then again evacuated and completely immersed in a water-bath at 37°C. After 2 hr the reaction was stopped by tipping the H_2SO_4 into the enzyme mixture. The H_2S evolved on gently rocking the tube was absorbed in the NaOH, which was then transferred to a 25-ml volumetric flask and the sulphide content estimated by the formation of methylene blue after a slight modification of the method of Fogo and Popowsky (1949). The relationship between optical density at 670 $m\mu$ and amount of H_2S appeared to be linear up to about 25 μg H_2S (Fig. 2). In estimations of enzyme activity variations of about ± 5 per cent. from the mean were observed.

The insects used in this work were taken from laboratory cultures of *Tineola bisselliella* (Humm.) larvae, *Ephestia kühniella* Zell. larvae, *Tribolium confusum* Duv. larvae and adults, *Rhizopertha dominica* (F.) adults, *Calandra granaria* (L.) adults, *Oryzaephilus surinamensis* (L.) adults, *Musca domestica* (L.) larvae, and *Anthrenus flavipes* Le Conte (= *A. vorax* Waterh.) larvae. Some *Coptotermes acinaciformis* (Froggatt) workers and adults of *Iridomyrmex* *detectus* (F. Sm.) were obtained from the field. Rats used in this work were white laboratory-bred rats from the Australian National University.

All insect enzyme solutions were prepared by grinding the fresh tissue, generally whole insects, with sand and distilled water in a mortar. After centrifugation and removal of fat, all solutions were held at about −20°C, usually in small portions which were used once, then discarded. Samples of $CHCl_3$-treated desulphydrase were prepared according to the method of Smythe (1942).

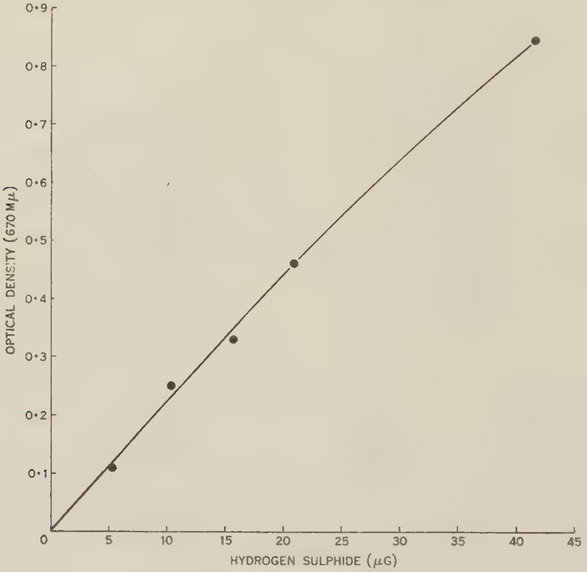

Fig. 2.—Relationship between optical density at 670 mμ and amount of hydrogen sulphide in 25-ml volume.

III. Experimental

(*a*) *General Properties of the Insect Enzymes*

The effects of reaction time and enzyme concentration on the yield of H_2S are shown in Figure 3*A*, *B*. Figure 3*C* represents the variation in the initial reaction rate with substrate concentration and these values are plotted in the reciprocal form of Lineweaver and Burk (1934) in Figure 3*D*. The Michaelis constant (K_m) derived from the slope and intercept of this line is equal to $3 \cdot 8 \times 10^{-2}$M.

Crude or chloroform-treated preparations of *Tineola* desulphydrase lost activity when stored at room temperature or even in the refrigerator, but acetone powders and solutions stored at sub-zero temperatures were relatively stable. High temperatures destroyed the enzyme very rapidly (Table 1).

The effect of pH on the activity of insect enzymes was studied by using 0·2M *l*-cysteine as substrate. In preliminary tests with lower concentrations of cysteine (0·0083M) buffered with phosphate, borate, glycine, histidine, or veronal considerable variations in enzyme activity were observed, depending upon the buffer used. When tested for 2 hr at 37°C on a final concentration of 0·2M *l*-cysteine the insect enzymes studied were found to have the following optima: *T. bisselliella* pH 8·9, *E. kühniella* pH 8·7, *C. granaria* pH 8·6, *A. flavipes* pH 8·3, *M. domestica* pH 7·7 (Fig. 4).

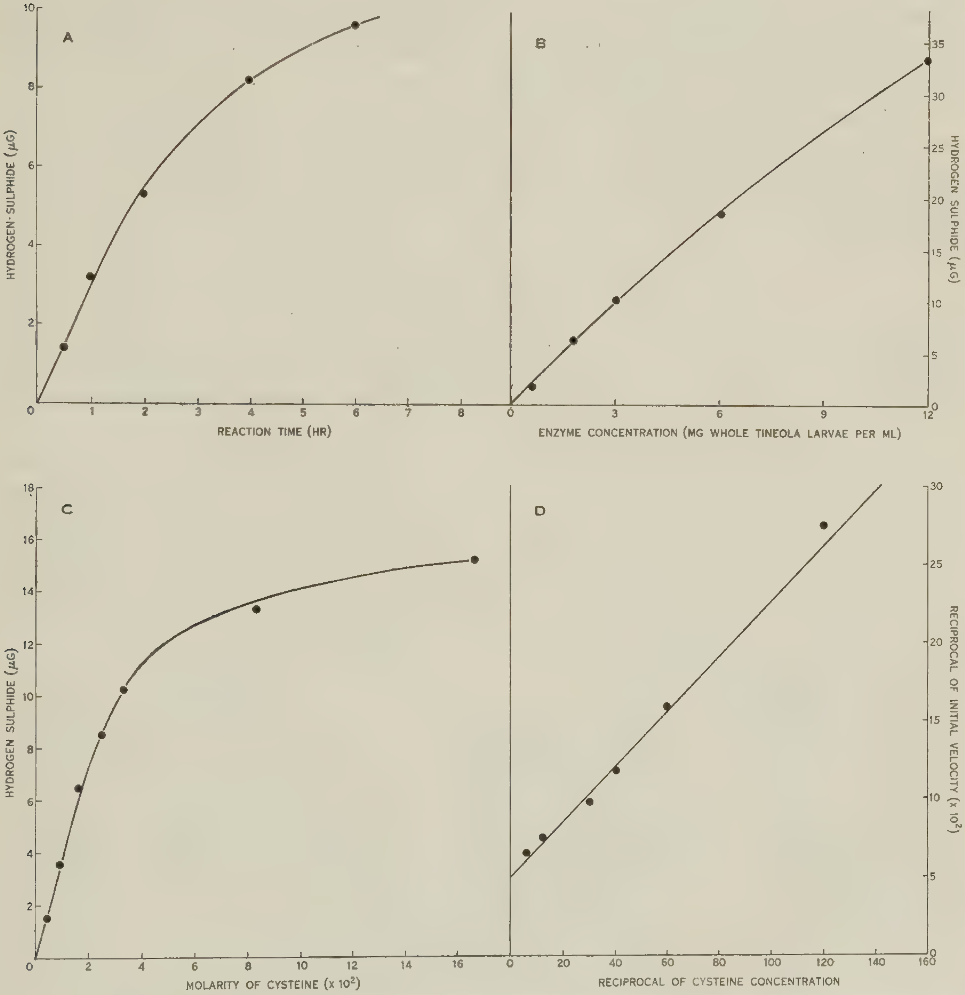

Fig. 3.—Effect of enzyme and substrate concentration and reaction time on the activity of *Tineola* desulphydrase. *A*, enzymic breakdown of cysteine at 37°C and varying times: 0·2M *l*-cysteine at pH 8·9. Buffered enzyme extract from 1·8 mg whole larval tissue. Final volume 2·4 ml. *B*, enzymic breakdown of cysteine in 2 hr at 37°C and varying enzyme concentrations: 0·2M *l*-cysteine, pH 8·9. Final volume 2·4 ml. *C*, enzymic breakdown of cysteine in 2 hr at 37°C and varying cysteine concentrations. Aqueous enzyme extract from 33 mg whole larval tissue. Final volume 2·4 ml. *D*, transposition of Figure 3*C*.

Estimations of desulphydrase in *Tineola* larval extracts showed that the activity varied considerably with the extraction technique. The more usual figure was about 400-600 μg H_2S per g tissue wet weight, although a figure of

4250 μg H_2S per g tissue was obtained for one ice-cold buffered extract. Most of the activity was to be found in the gut, one test yielding an activity of 1250 μg H_2S per g in the gut and only 78 μg H_2S per g in the remainder of the larval body. Comparative tests, using the same technique throughout, showed that *Tineola* larvae had a rather higher desulphydrase activity than a variety of other insects, including *A. flavipes* (see Fig. 4). Adults of *O. surinamensis*, workers of *C. acinaciformis*, larvae and adults of *T. confusum*, and adults of *I. detectus* had little or no desulphydrase activity.

Methionine, homocystine, homocysteine, and thioglycollate did not function as substrates with insect preparations and glutathione yielded an extremely small amount of H_2S. Neither H_2S nor cysteine (nitroprusside test) were obtained from cystine.

Table 1

STABILITY OF *TINEOLA* DESULPHYDRASE

All reaction mixtures contained 0·1-1·0 ml enzyme solution, depending upon the activity, a final concentration of 0·2M *l*-cysteine at pH 8·9 in total vol. 2·4 ml held at 37°C for 2 hr. The dialyses were carried out at 0°C for 18 hr with 500 volumes of distilled water, or of 0·01M glycine-NaOH buffer at pH 8·9

Preparation	Treatment	Activity (μg H_2S/ml enzyme)		Decrease in Activity (%)
		Original	*Final*	
Crude *A* (in water) ..	Deep freeze 18 hr	62	61	2
Crude *B* (in buffer pH 8·9)	Deep freeze 18 hr	176	174	0
Crude *A* (in water) ..	Refrigerator 18 hr	62	53	15
Crude *B* (in buffer pH 8·9)	Refrigerator 18 hr	176	158	10
Crude *A* (in water) ..	Dialysed against water	62	17	72
Crude *B* (in buffer pH 8·9)	Dialysed against buffer pH 8·9	176	141	20
Crude *C* (in water) ..	Heated 5 min at 50°C	8·8	3·3	63
Crude *C* (in water) ..	Heated 5 min at 60°C	8·8	0	100
Crude *C* (in water) ..	Refrigerator 9 days	80	30	62
$CHCl_3$-treated *D*	Refrigerator 9 days	23	3·3	85
Acetone-dried powder *E*	Extracted after 9 days at 0°C	10·7	9·8	10
Extract of powder *E* ..	Refrigerator 9 days	10·7	3·4	68

(*b*) *Effects of Various Activators and Inhibitors*

A group of substances, including some carbonyl reagents, was tested on insect preparations (Table 2). All the insect preparations were inhibited strongly by 10^{-2}M As_2O_3 and 10^{-2}M phenylhydrazine, and *Tineola* enzyme was also strongly inhibited by 10^{-2}M hydroxylamine but not by 10^{-2}M semicarbazide. Preparations of *M. domestica*, *C. granaria*, and *E. kühniella* were strongly inhibited by 10^{-2}M cyanide but *A. flavipes* was not affected and *T.*

bisselliella was strongly activated. Other experiments showed that the activation of *Tineola* enzyme occurred also with lower concentrations of cysteine as substrate irrespective of whether the buffer was composed of glycine, veronal, phosphate, borax, or histidine.

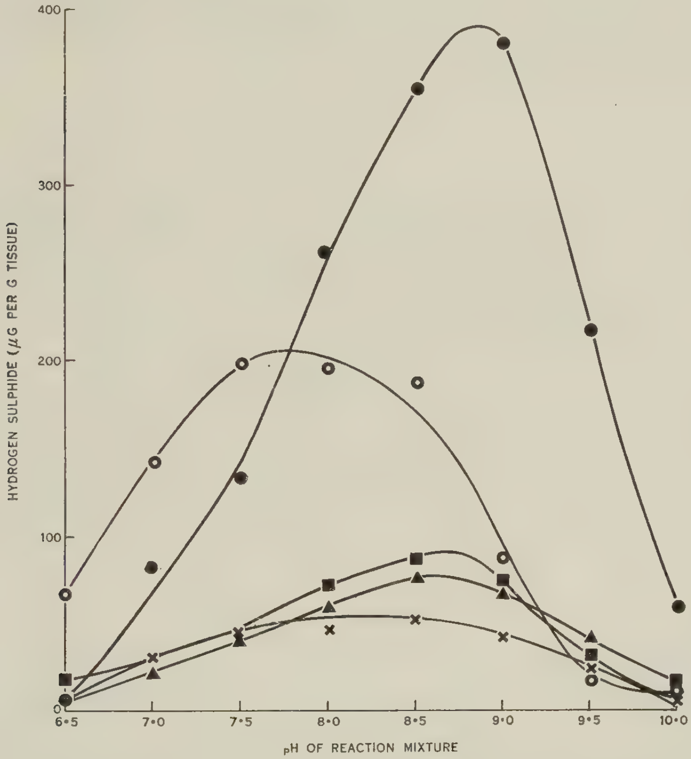

Fig. 4.—Effect of change of pH on desulphydrase in various insects. Reaction mixtures: 0·5 ml enzyme solution, 0·2M *l*-cysteine in final volume 2·4 ml, 2 hr reaction period at 37°C.
●–●, *Tineola bisselliella,* larvae.
O–O, *Musca domestica,* larvae.
■–■, *Ephestia kühniella,* larvae.
▲–▲, *Calandra granaria,* adults.
X–X, *Anthrenus flavipes,* larvae.

Relatively concentrated solutions of inorganic salts, i.e. 0·42M sodium chloride or potassium nitrate, inhibited all the insect enzymes tested (Table 2), but this inhibition was not as marked when a lower concentration of substrate buffered with glycine was used. Sodium fluoride (10^{-2}M) affected the insect enzymes only slightly, the maximum effect being 14 per cent. inhibition on *Anthrenus* desulphydrase. Sodium bisulphite (10^{-2}M), which is in the form of sulphite at the pH used, inhibited the *Tineola* enzyme strongly (Table 2). Two compounds known to combine with metallic ions, i.e. thiourea and sodium pyrophosphate (10^{-2}M), had little effect on the insect preparations.

The effect of sulphur-containing compounds on desulphydrase activity is shown in Table 2. Glutathione (10^{-1}M) and methionine (5×10^{-2}M) did not inhibit the *Tineola* preparation but slightly inhibited the other enzymes. When a lower concentration of substrate (0·0083M cysteine) was used 0·0083M methionine had no effect on *Tineola* enzyme but 0·0083M glutathione produced a 67 per cent. inhibition. Under the same conditions 0·0083M thioglycollate inhibited *Tineola* enzyme to the extent of 32 per cent. Table 2 shows also that 10^{-2}M serine has no effect on *Tineola* preparation.

TABLE 2

EFFECTS OF VARIOUS COMPOUNDS ON INSECT DESULPHYDRASE

Reaction mixture: 0·5 ml enzyme, 0·5 ml reagent solution, total volume 2·4 ml, final concentration *l*-cysteine 0·2M. All solutions adjusted to optimum pH for enzyme action and held at 37°C for 2 hr. The enzyme solutions had the following activities in μg H_2S per ml liberated under the above conditions. *Tineola* 16; *Anthrenus* 11; *Musca* 29; *Calandra* 32; *Ephestia* 18

Compound	Change in Activity of Enzymes (%)				
	Tineola	*Anthrenus*	*Musca*	*Calandra*	*Ephestia*
KCN 10^{-3}M	+33	+3	−6	−1	−4
KCN 10^{-2}M	+63	0	−38	−31	−33
As_2O_3 10^{-2}M	−47	−65	−71	−54	−38
Phenylhydrazine 10^{-2}M	−24	−52	−30	−51	−69
NaCl 0·42M	−43	−55	−63	−43	−54
KNO_3 0·42M	−73	−75	−88	−75	−74
Thiourea 10^{-2}M	+5	+4	+1	+3	0
$Na_4P_2O_7$ 10^{-2}M ..	−6	−19	0	−6	−10
NaF 10^{-2}M	−6	−14	−10	−12	−4
Glutathione 10^{-1}M ..	+4	−23	−30	−14	0
Methionine 5×10^{-2}M	0	−2	−4	−17	−19
Semicarbazide 10^{-2}M ..	−6				
Hydroxylamine 10^{-2}M ..	−52				
$NaHSO_3$ 10^{-2}M ..	−84				
Serine 10^{-2}M	−7				

(*c*) *Effect of Possible Coenzymes*

An aqueous extract of *Tineola* larvae lost 72 per cent. of its desulphydrase activity on dialysis against distilled water overnight in the refrigerator. Thirteen per cent. of activity was lost in an undialysed control, and the net effect of dialysis was therefore about 60 per cent. loss of activity (Table 1). An extract of *Tineola* larvae in 0·01M glycine-NaOH buffer, pH 8·9, lost only a net 10 per cent. of activity when dialysed against the same buffer overnight in the refrigerator. No reactivation of dialysed *Tineola* desulphydrase was observed on addition of a boiled undialysed extract, or of low concentrations of Mg, Mn, or Zn salts. In fact, Mg and Zn at 2×10^{-2}M were inhibitory.

Table 3

ATTEMPTED REACTIVATION OF DIALYSED *TINEOLA* EXTRACTS

Reaction mixture: 0·5 ml dialysed *Tineola* extract, 0·5 ml solution of Mn^{++}, Mg^{++}, or Zn^{++}, or boiled undialysed enzyme, 0·2M *l*-cysteine in final volume of 2·4 ml, pH 8·9. Held at 37°C for 2 hr. Activity of the *Tineola* enzyme solution under the above conditions equals 18·2 μg H_2S per ml

Additive	H_2S (μg)	Change in Activity (%)
0	9·1	
$MnSO_4$ 2×10^{-2}M	4·1	−55
$MgSO_4$ 2×10^{-2}M	9·0	0
$ZnSO_4$ 2×10^{-2}M	2·7	−70
Boiled undialysed enzyme	9·1	0

A dialysed extract of rat liver was activated on addition of pyridoxal phosphate or of ATP and either pyridoxal or pyridoxamine (Table 4). When dialysed *Tineola* extract was tested in the same type of experiment, it was found that pyridoxal or pyridoxamine alone inhibited the enzyme; however, when the vitamin B_6 derivatives and ATP were mixed no inhibition or activation was observed. The addition of pyridoxal phosphates to dialysed *Tineola* preparations also had no effect on their desulphydrase activity (Table 4).

Table 4

EFFECTS OF VITAMINS AND THEIR DERIVATIVES ON DESULPHYDRASE

Reaction mixture: 0·5 ml enzyme solution (dialysed against water 18 hr at 0°C), buffer and substrate as under, additives as shown; total volume 2·4 ml. Held at 37°C for 2 hr. The reaction mixture for the rat-liver and the *Tineola A* experiments contained 1 ml M/10 veronal-HCl buffer at pH 7·4 and 8·9 respectively and a final concentration of $8 \cdot 6 \times 10^{-2}$M *l*-cysteine. The *Tineola B* experiment contained only a final *l*-cysteine concentration of 0·2M at pH 8·9. Original activity of enzymes in μg H_2S/ml was: rat liver = 5·4; *Tineola* expt. *A* = 2·1; expt. *B* = 20

Additive	Change in Activity (%)		
	Rat Liver	*Tineola*	
		Expt. *A*	Expt. *B*
ATP alone (2 mg)			0
ATP (2 mg) + 0·2 mg pyridoxal	+90	+5	−2
ATP (2 mg) + 0·2 mg pyridoxamine	+59	−9	−2
Pyridoxal alone (0·2 mg)	0	−79	−55
Pyridoxamine alone (0·2 mg)	−8	−88	−70
Pyridoxal phosphate (0·002 mg)	+39		+2
Folic acid (0·2 mg)			−2

IV. Discussion

Tineola larvae have a very active cysteine desulphydrase which is concentrated mostly in the gut. Extracts of some other insects and of rat liver appeared to be less active than those of *Tineola,* and some insects had no desulphydrase activity. It has been shown that the desulphydrases from the various insects tested have their pH optima in the alkaline range between pH 7·7 and 8·9, this being higher than that for the rat liver desulphydrase (about pH 7·4-7·8, Smythe 1942).

Although a Michaelis constant has not been published for other desulphydrases, Lawrence and Smythe (1943) have illustrated the relationship between rat liver desulphydrase and cysteine concentration under their conditions. Calculations from their published graph yields a figure of approximately $4 \cdot 6 \times 10^{-2}$M. It appears that there is a close similarity between rat liver and *Tineola* larval enzymes in this respect.

Waterhouse (1952*a,* 1952*b*) has shown that heavy metals such as mercury, copper, cobalt, etc., when given in the food, are excreted as sulphides by the clothes moth (*Tineola*) but not by the carpet beetle (*Attagenus*). It is possible that the desulphydrase in the *Tineola* larva may play a part in this detoxification mechanism. The relatively weak desulphydrase in carpet beetles is interesting in view of the lack of sulphide formation in these insects. The difference in desulphydrase activity between these two groups of keratin-digesting insects may also be correlated with the low cystine excretion in *Tineola* compared with that of *Attagenus piceus* (Powning 1953).

Laskowski and Fromageot (1941) found that crude dog liver desulphydrase was rather unstable since it lost activity rapidly on heating at 50°C and pH 6, and even on storage at 4°C. They also found that an acetone-dried powder was stable. These properties are matched closely by *Tineola* preparations (Table 1).

Neither Smythe (1942) nor Fromageot, Wookey, and Chaix (1941) found any large decrease of activity after dialysis of rat or dog liver desulphydrase; however, Binkley (1943) found a strong inhibition on dialysing preparations from microorganisms or mouse liver. A crude aqueous extract of *Tineola* desulphydrase lost considerably more activity on dialysis against water than when dialysed against buffer at pH 8·9. It appears likely that the loss of activity in water is due to denaturation of the enzyme. Vitamin B_6 derivatives have been shown by other workers to function as coenzymes in the desulphydrase systems of rat liver and microorganisms (Braunstein and Azarkh 1950; Kallio 1951; Azarkh and Gladkova 1952). However, the desulphydrase of *Tineola* larvae, after dialysis against water, is not activated by these compounds (see Table 4). It is possible that specificity exists for certain vitamin B_6 derivatives, i.e. pyridoxamine phosphate may be required instead of pyridoxal phosphate. The reverse of this has been shown to be true of coracemase activity in *Streptococcus faecalis* (Wood and Gunsalus 1951) in which pyridoxamine phosphate is inactive. No pyridoxamine phosphate was available; however, the test with pyridoxamine and ATP was negative.

It appears that even the 10 per cent. activity lost on dialysis against buffer is probably not due to the loss of a dialysable B_6 derivative. It is likely that if a coenzyme is required for this enzyme it is firmly attached to the apoenzyme and is not removed by dialysis. Mouse liver preparations suffered inhibition on dialysis but this effect was reversed on addition of 10^{-3}M Mn, Zn, or Mg ions; further inactivation followed addition of 10^{-3}M sodium fluoride (Binkley 1943). Slightly higher concentrations of Mn and Zn ions (2×10^{-2}M) than those used by Binkley were found to further inhibit dialysed *Tineola* preparations, although the same concentrations of Mg ions and sodium fluoride had no effect. In view of these results it is interesting that Smythe (1942) has discussed the possible role of Mg as a component part of rat liver desulphydrase.

An activation of desulphydrase by folic acid was described by Binkley, Christensen, and Jensen (1952). No activation was found with liver or bacterial preparations (Azarkh and Gladkova 1952), nor in the present work in dialysed rat or *Tineola* preparations.

Low concentrations of various compounds known to react with carbonyl groups cause a strong inhibition of rat liver desulphydrase (Lawrence and Smythe 1943). This was found to be true in general also of five insect preparations using 10^{-2}M phenylhydrazine, but *Tineola* extract was little affected by 10^{-2}M semicarbazide, also a carbonyl reagent. No explanation can be offered at this stage for the behaviour of the enzymes with 10^{-2}M potassium cyanide, which strongly activated *Tineola* desulphydrase but inhibited other insect enzymes as well as rat liver preparations. The effects of compounds forming metal complexes, inorganic salts in high concentration, and the possibility of a competitive inhibition by glutathione and thioglycollate show similarities between insect and rat liver preparations (see Lawrence and Smythe 1943).

Rat liver extracts have been shown to liberate H_2S from cystine at the same rate as from cysteine, and it was suggested that cysteine and sulphur could be end-products of this reaction (Smythe 1942). It was thought that if this reaction occurred in keratin-digesting insects it might be of importance in the primary breakdown of the disulphide bonds of keratin. It has been shown, however, that insects do not liberate H_2S from methionine, thioglycollate, or homocysteine and that neither H_2S nor cysteine are obtained from cystine. Possibly there was some reducing material present in the rat liver extracts which did not occur in the insect extracts.

V. Acknowledgments

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A STUDY OF THE ACTION OF PURIFIED THYROID PROTEASE ON ^{131}I-LABELLED THYROGLOBULIN

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Summary

The preparation of pig thyroglobulin labelled with ^{131}I is described and the results of the action of purified thyroid protease on different samples are presented. The major iodine-containing compounds liberated were found to be mono- and di-iodotyrosine, thyroxine, and tri-iodothyronine, together with a "peptide" fraction; of these mono-iodotyrosine, di-iodotyrosine, and the "peptide" predominated. Specific activities of these components were determined and compared with the values obtained following alkaline hydrolysis of the labelled protein.

A radioactive spot of intensity similar to that of thyroxine and of slightly lower R_F value in the solvent system used was also observed. Its identity has not yet been established.

Preliminary experiments with labelled synthetic iodopeptides as substrates indicate that the protease possesses peptidase activity at acid pH which may be preferentially concerned with the fission of bonds joining aromatic amino acid residues; the thyroid enzyme would thus behave, in this respect, similarly to pepsin.

I. Introduction

The application of the radioiodine technique has contributed much in recent years to our knowledge of the processes underlying thyroid function both from the experimental and clinical angles. Not only does it enable an assessment to be made of the level of thyroid function *in vivo* but, in association with partition chromatography and allied techniques, the unravelling of many of the details of the biochemical reactions involved has been accomplished using comparatively small amounts of material.

The investigations described in this paper have been concerned with the action of a purified protease isolated from the thyroid gland (McQuillan and Trikojus 1953) on thyroglobulin biologically labelled with ^{131}I. These studies are an extension of those forming the subject of a preliminary report to the Oxford Isotope Conference, 1951 (Gordon *et al.* 1953). Roche and colleagues (Roche *et al.* 1950; see also Michel 1952) have also investigated the proteolysis of labelled thyroglobulin, using unpurified glycerol extracts of thyroid tissue as the source of enzyme. In several important aspects, our results have been in line with those of the French workers.

In order to understand further the mechanisms involved, an investigation of the action of the purified enzyme on synthetic ^{131}I-labelled and other peptides has been initiated and some preliminary results are also included in this paper.

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II. Materials and Methods

Thyroid protease.—This was prepared and tested according to McQuillan and Trikojus (1953). The enzyme preparations used had proteolytic activities in the range 25-40 units*/mg.

Radioiodine.—This was supplied in solution as $Na^{131}I$, carrier-free.

Labelled thyroglobulin was prepared from the thyroids of pigs which had been injected with $Na^{131}I$ and was purified as described in the experimental section. Since it was found (McQuillan and Trikojus 1953) that thyroglobulin purified in this manner still retained proteolytic activity, each preparation was tested, using haemoglobin as substrate, as for purified thyroid protease. As the rate of reaction of the protease with haemoglobin follows a straight line over 2 hr, the period of incubation at 37°C was extended to 90 min in order to conserve the labelled thyroglobulin.

Iodoamino acids, which served to identify spots on the chromatograms, were recrystallized samples obtained by standard methods. A specimen of synthetic 3,5,3′-tri-iodothyronine was kindly provided by Glaxo Pty. Ltd. (England); one of the same substance labelled in the 3′-position was prepared from 3,5-di-iodothyronine by the micro-iodination procedure of Lemmon, Tarpey, and Scott (1950). *The peptides* used were synthetic samples as indicated in the text.

Iodine (${}^{127}I$) values were determined by the method of Barker (1950).

pH.—Throughout the work, pH was checked with the glass electrode.

Chromatography.—Solvents for use in chromatography were purified by standard procedures. Solvent systems used were: phenol-water with an atmosphere of ammonia (Partridge 1948); *n*-butanol-*n*-pentanol-ammonia in the volume proportions 1 : 1 : 2; *n*-pentanol-propionic acid-water in the volume proportions 20 : 3 : 15. These systems are subsequently referred to in the text as PWA, BPA, and PPW respectively.

Radioactivity was measured by standard methods. Solutions, after suitable dilution, were assayed in M12, M6, or M6H Veall tubes (20th Century Electronics) by comparison with standard solutions of known radioactivity. In certain instances chromatograms of extracts of enzyme-treated labelled thyroglobulin, prepared on washed No. 1 or No. 3 Whatman paper, were scanned, after removal of the solvent, at 1 cm intervals using 1 min counts; the papers were held in a lead-shielded carrier fitted at the top level of the "Perspex" frame of a standard lead castle under a GM4 tube (Oddie and Mibus 1949); more usually radioautograms were obtained by contact with "No-screen" X-ray film ("Kodirex" or "Ilfex").

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* One unit is the amount of enzyme required to liberate 10^{-4} m-equiv. tyrosine in 30 min at 37°C (haemoglobin substrate).

III. Experimental Procedure

(*a*) *Preparation of ^{131}I Thyroglobulin*

Young pigs weighing 17-40 kg were injected subcutaneously in the abdominal region with measured doses of purified carrier-free ^{131}I. Twenty-four hr later the animals were killed by bleeding from the shoulder. The thyroid glands, after clean dissection, weighing, and sampling, were thinly sliced in the frozen state and extracted twice with saline (3 vol. and 1 vol. respectively). The extract was then put through a single fractionation run by the method of Derrien, Michel, and Roche (1948), modified in that the equilibration period at each concentration of ammonium sulphate was shortened from 12 to 5 hr. The 37-41 per cent. fraction was rapidly dialysed and freeze-dried. The distribution of ^{131}I was followed throughout, the various fractions being dissolved, where necessary, in 2N NaOH before counting. Results obtained in seven preparations together with values for ^{127}I content and proteolytic activity of the final products are listed in Table 1. The more reliable uptake figures recorded in this table are considered to be those calculated in each case from the sum of the radioactivity values of the individual fractions; those derived from the small assay samples can only be approximate.

(*b*) *Preparation of Enzyme Digests and Extracts*

Aqueous solutions of thyroglobulin and of protease at pH 3·5 were prepared by adjustment with 2N or 0·5N acetic acid. The requisite volumes of substrate and of enzyme were then mixed, toluene added as preservative, the tubes stoppered and shaken at 37°C (usual period of incubation 24 hr). Experiments were usually controlled in two ways: (i) by a sample containing added enzyme maintained at 0°C during the incubation period; (ii) by one in which the added enzyme was inactivated by heating at pH 3·5 and 100°C for 15 min before mixing with the solution of thyroglobulin. At the conclusion of the incubation period, ethanol was added to 70 per cent. concentration and the precipitate, which was readily flocculated by the addition of a micro-drop of conc. NH_4OH, was removed by centrifuging and washed twice with minimum amounts of 70 per cent. ethanol.

(*c*) *Chromatography of Extracts*

An extract to be chromatographed was evaporated to dryness under reduced pressure and the residue dissolved in the minimum quantity of a solution composed of ethanol:conc. NH_4OH:water in the proportions 25 : 1 : 25. This material was then applied to washed Whatman No. 1 or No. 3 paper either as a spot or, more frequently, as a continuous line, particularly when relatively large amounts of material were to be chromatographed. After comparison with the corresponding radioautogram, the radioactive areas were traced on the paper strips, these sections were then cut into small pieces and eluted overnight at 0°C with 0·1N NH_4OH plus a few drops of toluol. The eluates were then treated as were the initial extracts and rechromatographed either in the same or in another solvent system. Radioactive components on chromatograms were identified by coincidence in position and shape with ninhydrin-reacting spots which were formed from authentic iodoamino acids added to the extract before chromatography; when specific activities were to be determined, the mixture of unlabelled iodoamino acids was applied in an adjacent position. R_F values are given singly, from the centres of small discrete spots, or as a range to define the limits of larger diffuse areas.

IV. Results

The results of preliminary experiments indicated that there is a well-marked action of the protease on thyroglobulin leading to the liberation of 3-iodotyrosine, 3,5-di-iodotyrosine, thyroxine (in small amount), and peptides containing iodoamino acids, apart from iodine-free fragments. Later experiments, involving larger amounts of labelled thyroglobulin of higher specific activity, were designed to obtain more quantitative data and additional information as to the products of hydrolysis. Details of the experiments using the last three preparations of thyroglobulin (*RA*6, *RA*13, *RA*21; see Table 1) are reported below.

(*a*) *Experiment* 1. *Thyroglobulin Preparation* RA6 *and Enzyme Preparation* E17 (39 *"units"/mg*)

The bulk of the labelled thyroglobulin was submitted to the action of the protease as follows:

	Thyroglobulin	Enzyme	Treatment
E_{37}	253·3 mg; 16·0 ml	32 mg; 3·2 ml	37°C; 24 hr
e_{37}	15·8 mg; 1·0 ml	2 mg; 0·2 ml	37°C; 24 hr
Control (*C*)	15·8 mg; 1·0 ml	2 mg; 0·2 ml (heat-inactivated)	37°C; 24 hr

The digest (e_{37}) was included in order to effect a more exact comparison with the control (*C*). Following incubation, proteins were precipitated at room temperature and the radioactive contents of the 70 per cent. ethanol supernatants and residues determined. Results are set out in Table 2. It is apparent that the ^{131}I contents of the ethanol supernatants of the test samples, containing added active enzyme, were more than twice that of the control, where the radioactivity of the 70 per cent. ethanol-soluble extract was presumably due to the action of enzyme residual in the thyroglobulin substrate (for enzyme activities of the thyroglobulin preparations see Table 1).

(i) *Treatment of Ethanol Extracts.*—Enzyme digest (*E*) was applied to a number of paper sheets and chromatographed in phenol-water-ammonia (PWA); the amount applied was 76·6 μc. Three radioactive zones were eluted (with centres of maximum intensity at R_F 0·63 (*L*), 0·81 (*M*), and 0·94 (*H*)—

the radioactivity recovered being 40·3, 10·9, and 3·18 μc respectively). The eluates were then re-run in PWA. Since further investigation indicated that the zone of radioactivity at R_F 0·94 was an artefact (possibly by phenol-iodine interaction) this material was discarded. Radioactive zones *L* and *M* were again cut out, care being taken to exclude mutual contamination, and eluted. These eluates contained 24·4 and 7·69 μc respectively, representing recoveries of approx. 80 per cent. of material applied. They were evaporated to small volumes and one-third of each was taken for immediate chromatography in *n*-butanol-*n*-pentanol-ammonia (BPA) (see below). To each remaining two-thirds an equal volume of 4N NaOH was added and the alkaline solutions obtained then divided into two equal parts: one-half was maintained at 0°C, the other at 105°C for 16 hr. At the conclusion of this period, the alkaline solutions were extracted three times with an equal volume of butanol, and the butanol extracts, after partitioning three times against water (to reduce the ionic content), were counted and chromatographed in BPA. Water used for washing the butanol extracts was adjusted to pH 10 with NH_4OH.

In order to isolate di-iodotyrosine and related compounds the residual solutions resulting from the above procedure were adjusted to pH 3·5 with 2N HCl, and butanol extraction, washing (with water at pH 3·5 (HCl)), radioactivity estimation, and chromatography repeated as above.

The main chromatographic findings but not the quantitative results are presented since it was found that the water washings of the butanol extracts contained a high proportion of the initial activity of these extracts. The procedure was, therefore, modified in subsequent experiments.

(ii) *Chromatographic Identification of the Components of Zones* L *and* M.—The results obtained by chromatography of the purified zones before and subsequent to alkali treatment (at 0° and 105°C) established the presence of mono-iodotyrosine, di-iodotyrosine, thyroxine, tri-iodothyronine, and two unknown *n*-butanol-soluble components which migrated more rapidly than thyroxine in the solvent system BPA (Plate 1, Figs. 1, 2, and 3). The position occupied by tri-iodothyronine corresponded to that observed in earlier work by Hird and Trikojus (1948), using a similar solvent system. Presence of peptide material was suggested by the increase in intensity of the thyroxine and of the unidentified spots following alkaline hydrolysis. The two unknown radioactive spots in Plate 1, Figure 2, have been shown in later work (Stanley 1953) to be artefacts formed from di-iodotyrosine (and mono-iodotyrosine) when their alkaline solutions are heated.

In Experiments 2 and 3, initial resolution with BPA followed by rechromatography of the individual fractions in either BPA or PPW (pentanol-propionic acid-water) was substituted for the preliminary chromatographic separation in PWA as used in Experiment 1. Specific activities (μc ^{131}I/μg ^{127}I) of the main ethanol-soluble components of the enzyme digests were also determined and compared with the values obtained for these compounds following alkaline hydrolysis of the same sample of labelled thyroglobulin.

TABLE 1

LABELLED PIG THYROGLOBULIN (^{131}I) PREPARATIONS. SUMMARY OF DISTRIBUTION OF ^{131}I BETWEEN FRACTIONS AND OTHER RELEVANT DATA

	*RA2**		*RA3*		*RA4*		*RA5*		*RA6**		*RA13*		*RA21*	
	μc	%†	μc	%	μc	%	μc	%	μc	%	μc	%	μc	%
$Na^{131}I$ injected	(*a*) 500; (*b*) 521		488		505		500		(*a*) 743; (*b*) 743		1050		969	
Distribution in Thyroid Fractions:														
Assay samples	1·7		2·1		5·0		0·6		3·1		7·3		6·6	
Saline residues	9·9	8·1	12·7	7·8	10·4	8·0	4·6	5·7	62·0	11·3	15·3	10·0	30·2	13·1
Supernatant (42% $(NH_4)_2SO_4$)	9·7	7·9	15·1	9·2	5·7	4·4	2·1	2·7	20·5	3·7	12·5	8·2	6·5	2·8
Precipitate (37% $(NH_4)_2SO_4$) ..	32·2	26·3	40·4	24·7	38·8	29·9	18·1	22·5	94·1	17·2	19·0	12·4	49·1	21·5
Supernatant (41% $(NH_4)_2SO_4$)	6·1	5·0	13·5	8·3	10·6	8·2	0·6	0·7	49·0	8·9	25·0	16·4	11·9	5·2
Thyroglobulin (37-41% $(NH_4)_2SO_4$)	64·8	52·7	81·8	50·0	64·1	49·5	55·6	68·5	322·9	58·9	81·3	53·2	132·0	57·4
Total uptake														
(i) By addition	124·4	12·2	165·6	33·9	134·6	26·7	81·6	16·3	551·6	37·1	160·4	15·3	236·3	24·4
(ii) Calc. on assay sample ..	149		220		184		133		709		233		295	
Wt. pig (kg)	(*a*) 17; (*b*) 26		—		—		22		(*a*) 40; (*b*) 40		35		48	
Wt. of dissected thyroid gland (g)	(*a*) 1·95; (*b*) 2·47		1·70		4·36		2·41		(*a*) 3·49; (*b*) 3·46		2·95		3·07	
Wt. of thyroglobulin (Tg) (mg)	242		193		219		104		414		162		251	
^{127}I content of Tg (%)	0·86		0·34		0·75		0·48		0·40		0·52		0·77	
Proteolytic activity of Tg‡ ..	2·1		2·4		3·3		2·9		2·0		3·8		3·1	

* Two pigs injected. (In *RA2* the injections were made intraperitoneally, not subcutaneously; that given to the second animal was presumably lost in the bladder as the uptake was only 5·3 μc (calc. on assay sample). In *RA6* the respective uptakes were 372·4 and 336·3 μc.)

† With the exception of the values for total uptake, which are based on the quantities injected, the figures in the percentage columns relate to the contribution of the particular fraction to the total radioactivity recovered.

‡ Units expressed as m-equiv. × 10^4 of tyrosine liberated per mg thyroglobulin.

(*b*) *Experiment* 2. *Thyroglobulin Preparation* RA13 *and Enzyme Preparation* E22 (37 *"units"/mg*)

(i) *Enzymic Hydrolysis*

Enzymic hydrolysis of thyroglobulin was carried out as in Experiment 1. The following tests were set up:

	Thyroglobulin	Enzyme	Treatment
E_{37}	59 mg; 4·00 ml	12 mg; 1·2 ml	37°C; 24 hr
Control (E_0)	59 mg; 4·00 ml	12 mg; 1·2 ml	0°C; 24 hr
Control (C)	4·9 mg; 0·33 ml	1 mg; 0·1 ml (heat-inactivated)	37°C; 24 hr

At the conclusion of the 24-hr period, proteins were precipitated and the radioactivity of the 70 per cent. ethanol supernatants and residues determined. Results are recorded in Table 2. The fraction of the total radioactivity recovered in the 70 per cent. ethanol-soluble supernatant of the test sample maintained at 37°C (E_{37}) is seen to be about twice that of the control (C), with heated enzyme, and more than four times that of the control sample (E_0) maintained at 0°C.

Table 2

DISTRIBUTION OF RADIOACTIVITY FOLLOWING ETHANOL PRECIPITATION OF ENZYMIC DIGESTS OF LABELLED THYROGLOBULIN (EXPERIMENTS 1, 2. AND 3)

Test	Activity of Thyroglobulin Sample (μc)	Soluble in 70% Ethanol		Insoluble in 70% Ethanol		Total ^{131}I Recovered
		μc	%	μc	%	(μc)
Experiment 1						
Digest at 37°C (major sample)	197·3	79·32	38·1	129·0	61·9	208·3
Digest at 37°C (minor sample)	12·33	4·94	38·0	8·09	62·0	13·03
Control digest at 37°C (added enzyme heat-inactivated) ..	12·33	1·68	15·3	9·33	84·7	11·01
Experiment 2						
Digest at 37°C ..	29·7	12·3	37·5	20·5	62·5	32·8
Digest at 0°C ..	29·7	2·51	8·4	27·5	91·6	30·0
Digest at 37°C (added enzyme heat-inactivated)	2·45	0·48	20·4	1·87	79·6	2·35
Experiment 3						
Digest at 37°C ..	57·6	12·3	19·7	50·4	80·3	62·7

Treatment of ethanol extracts and chromatographic identification.—The three extracts were applied to paper and chromatographed in BPA. Five radioactive zones were apparent (at R_F 0·02 (I*A*), 0·07 (II*A*), 0·13 (III*A*), 0·16-0·30 (IV*A*), 0·38-0·48 (V*A*)) in the radioautogram of the E_{37} digest (see Plate 2, Fig. 4), whereas in the corresponding radioautograms of the controls (E_0 and *C*), the first three zones were well marked but no radioactivity was detected in the regions of R_F 0·16-0·30 and 0·38-0·48. However, chromatograms of all three samples were cut in a similar manner at the limits of the radioactive zones in E_{37} and the radioactivity, after elution, estimated. Results are set out in Table 3.

Table 3

RADIOACTIVITY OF VARIOUS ZONES ELUTED FROM CHROMATOGRAMS (*n*-BUTANOL-*n*-PENTANOL-AMMONIA) OF 70 PER CENT. ETHANOL EXTRACTS OF ENZYME DIGESTS (EXPERIMENTS 2 AND 3)

	μc	%*	μc	%	μc	%	μc	%	μc	%
Experiment 2	Zone I*A*† R_F 0·02		Zone II*A* R_F 0·07		Zone III*A* R_F 0·13		Zone IV*A* R_F 0·16-0·30		Zone V*A* R_F 0·38-0·48	
Digest at 37°C	4·11	63·1	1·48	22·7	0·22	3·38	0·44	6·75	0·26	3·99
Digest at 0°C	0·80	69·0	0·20	17·3	0·05	4·31	0·06	5·08	0·05	3·97
Digest at 37°C (added enzyme heat-inactivated)	0·16	66·6	0·03	13·8	0·03	10·4	0·01	4·16	0·01	5·00
Experiment 3	Zone I*C*† R_F 0-0·07		Zone II*C* R_F 0·09		Zone III*C* R_F 0·13-0·21		Zone IV*C* R_F 0·23-0·34		Zone V*C* R_F 0·42-0·52	
Digest at 37°C	4·48	58·6	1·72	22·5	0·73	9·6	0·35	4·6	0·36	4·7

* Expressed as percentage of total activity recovered.

† The approximate activity ratios of di-iodotyrosine to "peptide", the two components of this zone, in both Experiments 2 and 3 were, respectively, 51 : 49 and 45 : 55 (E_{37} digests).

Since the radioactivity was too low, the eluates of control (*C*) were not further investigated. Those of E_{37} and of the control (E_0) were rechromatographed and the identity of the major radioactive components established as follows:

Di-iodotyrosine (*DIT*) *and "Peptide."*—(1) E_{37}. The eluate of zone I*A* (R_F 0·02) of the original chromatograms was re-run in PPW. The radioautogram indicated activity at the origin, a second centre at R_F 0·31 (identified as DIT) with a trail between the main radioactive spots. DIT, the "trail," and the material at the origin were eluted. The specific activity of DIT was estimated from the corresponding eluate; the other two eluates were re-run in PPW. From the resultant chromatogram it was established that the trail consisted almost entirely of DIT; this amino acid was calculated to contain approx. 51 per cent. of the activity of the original zone I eluate. The material from the origin, presumably a peptide, was not resolved on re-running and was eluted for the estimation of its specific activity.

(2) E_0. From the first run in PPW of the eluate of zone IA, DIT, and "peptide" were eluted. The activity ratio of "peptide" to DIT (approx. 3 : 1) was appreciably greater than in E_{37} (approx. 1 : 1).

Mono-iodotyrosine (*MIT*).—E_{37} and E_0. Eluates of zone IIA (R_F 0·07) of the BPA chromatograms were run in PPW. Apart from MIT (R_F 0·25), only traces of radioactivity were detected. MIT was eluted and the specific activity of the E_{37} eluate determined.

Iodide.—E_{37} and E_0. Eluates of zone IIIA (R_F 0·13) were re-run in BPA. Iodide in small amount was identified as the principal constituent in each case.

Thyroxine (*Tx*).—Eluates of zone IVA (R_F 0·16-0·30) were re-run in BPA.

(1) E_{37}. Thyroxine was identified as the major constituent and its specific activity determined; traces of unidentified compounds were also present.

(2) E_0. A faint trace of Tx was detected and eluted.

Tri-iodothyronine (*TIT*).—Eluates of zone VA (R_F 0·38-0·48) were re-run in BPA.

(1) E_{37}. Radioactivity coincided with the R_F value given by a specimen of synthetic radioactive TIT. The spot was eluted and the specific activity estimated.

(2) E_0. A faint streak of radioactivity was detected in the same position as in E_{37}, but was not eluted.

The specific activities of identified compounds are listed in Table 5.

(ii) *Alkaline Hydrolysis of Thyroglobulin*

The labelled thyroglobulin sample *RA*13 (19·6 mg) was hydrolysed with 2N NaOH (6 ml) for 16 hr at 105°C. After adjustment to pH 3·5 with 2N HCl, the hydrolysate was extracted twice with an equal volume of *n*-butanol (which had been saturated with water at pH 3·5 (HCl)) and the butanol extract washed once with one-third its volume of water at pH 3·5. The butanol extract was found to contain 5·43 μc (84·3 per cent.), the aqueous residue 0·74 μc (11·5 per cent.), and the aqueous washings 0·27 μc (4·2 per cent.). The butanol extract was then chromatographed in BPA. Four major zones of radioactivity were detected in the radioautograms (R_F 0·01 (I*B*), 0·07 (II*B*), 0·12 (III*B*), 0·19-0·29 (IV*B*)); these were eluted and the ^{131}I contents determined. The results are given in Table 4.

Further treatment of the eluates of the four zones was carried out as with the E_{37} material in Experiment 2. DIT was recovered from zone I*B* (which also contained traces of MIT), MIT from zone II*B*, and iodide from zone III*B*.

Tx and TIT were tentatively identified in the first re-run in BPA of the eluate of zone IV*B*; they were eluted together and re-run in BPA. Tx only was eluted from this chromatogram.

Table 4

RADIOACTIVITY OF VARIOUS ZONES ELUTED FROM CHROMATOGRAMS (*n*-BUTANOL-*n*-PENTANOL-AMMONIA) OF *n*-BUTANOL EXTRACTS OF ALKALINE HYDROLYSATES OF LABELLED THYROGLOBULIN (EXPERIMENTS 2 AND 3)

	μc	%*	μc	%	μc	%	μc	%	μc	%
Experiment 2	Zone I*B* R_F 0·01		Zone II*B* R_F 0·07		Zone III*B* R_F 0·12		Zone IV*B* R_F 0·19-0·29		Zone V*B* —	
	1·56	50·8	0·52	16·8	0·51	16·8	0·48	15·6	—	
Experiment 3	Zone I*D* R_F 0-0·07		Zone II*D* R_F 0·09		Zone III*D*† R_F 0·12-0·18		Zone IV*D* R_F 0·20-0·28		Zone V*D* R_F 0·36-0·46	
	10·6	49·6	5·21	24·4	1·68 1·79	7·9 8·4	1·66	7·8	0·42	2·0

* Expressed as percentage of total activity recovered.

† Zone III*D* comprised two separately eluted bands of radioactivity, (*a*) and (*b*) respectively (cf. Plate 2, Fig. 6).

(*c*) *Experiment* 3. *Thyroglobulin Preparation* RA21 *and Enzyme Preparation* E35 (28·3 *"units"/mg*)

In this experiment the entire quantity of thyroglobulin was used for proteolysis at 37°C and for alkaline hydrolysis.

(i) *Enzyme Hydrolysis*

Thyroglobulin	Enzyme	Treatment
119·5 mg; 6·5 ml	26·9 mg; 3·05 ml	37°C; 24 hr

The subsequent treatment followed that used for E_{37} in Experiment 2.

(ii) *Alkaline Hydrolysis.*—Two separate hydrolyses were carried out with 30·1 mg and 30·8 mg thyroglobulin at 105°C for 15 hr; 6 ml 2N NaOH was used in each case. After hydrolysis the further treatment followed that used in Experiment 2 except that the pH was lowered to 2·7 prior to extraction with *n*-butanol, as this was found to give higher recoveries of labelled compounds.

After the first application to paper the eluates of the corresponding zones for each hydrolysate were combined.

The radioactivity distribution after ethanol precipitation of the enzyme digest is given in Table 2 and for the eluates of the primary chromatographic zones following enzymic and alkaline hydrolysis in Tables 3 and 4 (see also Plate 2, Figs. 5 and 6). The subsequent chromatographic resolution clearly distinguished the following as the major components of the primary zones indicated (specific activities are given in Table 5).

Table 5

SPECIFIC ACTIVITIES OF THE MAJOR ^{131}I-CONTAINING COMPOUNDS LIBERATED FROM LABELLED THYROGLOBULIN* BY THYROID PROTEASE AND BY ALKALINE HYDROLYSIS (EXPERIMENTS 2 AND 3)†

	Mono-iodotyrosine		Di-iodotyrosine		Thyroxine		Tri-iodothyronine		"Peptide"	
	Expt. 2	Expt. 3	Expt. 2	Expt. 3	Expt. 2	Expt. 3	Expt. 2	Expt. 3	Expt. 2	Expt. 3
Digest at 37°C (24 hr)										
^{131}I content (μc)	0·69	1·06	0·66	1·25	0·101	0·206	0·075	0·118	0·56	1·51
^{127}I content (μg)	5·3	12·4	5·9	7·9	0·84	4·2	1·7	3·2	6·4	23·0
Specific activity (μc/μg)	0·13	0·086	0·11	0·16	0·12	0·049	0·044	0·037	0·088	0·066
Alkaline hydrolysate										
^{131}I content (μc)	—	3·06	—	6·03	—	0·625	—	0·143	—	—
^{127}I content (μg)	—	26·4	—	70·0	—	12·0	—	2·9	—	—
Specific activity (μc/μg)	—	0·12	—	0·086	—	0·052	—	0·049	—	—

*Specific activities of thyroglobulin 0·096 μc/μg (Experiment 2) and 0·076 μc/μg (Experiment 3).

† It should be noted that the figures quoted do not necessarily represent total recoveries since the care taken to elute pure samples necessitated more than one chromatographic resolution and cutting of the radioactive areas within narrow limits.

(i) *Enzyme Hydrolysis.*—Zone I*C*: DIT, "peptide"; zone II*C*: MIT; zone III*C*: unknown, Tx; zone IV*C*: Tx; zone V*C*: TIT.

(ii) *Alkaline Hydrolysis.*—Zone I*D*: DIT, MIT (ratio of DIT : MIT approx. 4·6 : 1); zone II*D*: MIT; zone III*D* (*a* and *b*): I$^-$ and unknown (same R_F as unknown in III*C*), see also Plate 3, Figures 7 and 8; zone IV*D*: Tx; zone V*D*: TIT.

Zone II*C* contained in addition to MIT a small amount of radioactivity in a position subsequently shown to be occupied by an authentic sample of labelled mono-iodohistidine. This also appeared in zone III*D* of the alkaline hydrolysate between MIT and iodide. The unknown mentioned as occurring in the eluates of III*C* and III*D* was approximately of the same radioactive intensity as thyroxine. It could not have arisen as an artefact during alkaline hydrolysis as it was present in both types of hydrolysate; moreover the main alkali-produced artefacts observed by Stanley (1953), being of higher R_F, were deliberately excluded when the positions of the primary zones were defined. Identity with 3,5-di-iodothyronine was excluded on the basis of R_F comparison.

The small amounts of iodide found in the enzyme hydrolysates of Experiments 2 and 3 could have arisen in the original 70 per cent. ethanol extracts by elution of free iodide which had become adsorbed by the thyroglobulins during their preparation; alternatively the evaporation and chromatographic procedures associated with the resolution of the primary zones could have caused iodide fission from the iodine-containing components. Roche and colleagues (Roche *et al.* 1951) have demonstrated the presence of a deiodase in thyroid tissue acting specifically on MIT and DIT and have shown (Roche *et al.* 1952) that the enzyme is still active though unstable in a cell-free medium (pH optimum about 8·0). Our protease preparations were tested on labelled DIT at pH 3·5 and 7·5 and shown to be without deiodase activity.

(*d*) *Action of Thyroid Protease on Labelled Di-iodotyrosyl Peptides*

Three peptides were iodinated with ^{131}I plus carrier by the micro-procedure of Lemmon, Tarpey, and Scott (1950): L-leucyl-L-tyrosine (LT); glycyl-L-tyrosine (GT); *N*-acetyl-L-phenylalanyl-L-tyrosine (APAT). The first two were authentic samples from Roche Products Ltd. while the third was synthesized according to Baker (1951). The three iodinated reaction products were purified by paper chromatography in the solvent systems BPA (APAT) and PPW (LT and GT). From these chromatograms radioactive zones corresponding to the labelled di-iodotyrosyl peptides were eluted and purified by re-application to paper, using the same solvents except with iodinated APAT where phenol-water was found to achieve a better separation from contaminating iodide. The final eluates of ^{131}I peptides were evaporated to dryness *in vacuo* and each residue was dissolved in 0·5 ml acetate buffer, pH 3·2. Thyroid protease (activity 33·6 "units"/mg) and crystallized pepsin (Armour) were dissolved in the buffer to give concentrations of 5 mg/ml. The test solutions were prepared as shown in Table 6.

The tubes were incubated for 1 hr at 38°C and the reactions then retarded by rapid cooling to 0°C. To each tube unlabelled di-iodotyrosine was added as marker on the chromatograms, which were prepared with aliquots of the solutions and developed in a variety of solvent systems. The best resolutions were obtained with BPA on Whatman No. 3 paper run for 24 hr (using a pad of filter paper to absorb solvent at the bottom of the filter paper sheet). Radioautograms of the various digests are presented in Plate 3, Figure 9. Hydrolysis

ACTION OF THYROID PROTEASE ON THYROGLOBULIN

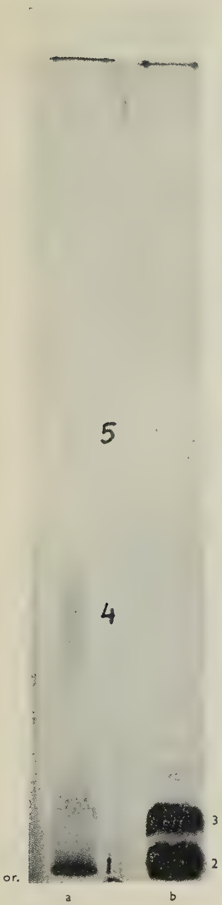


Fig. 1

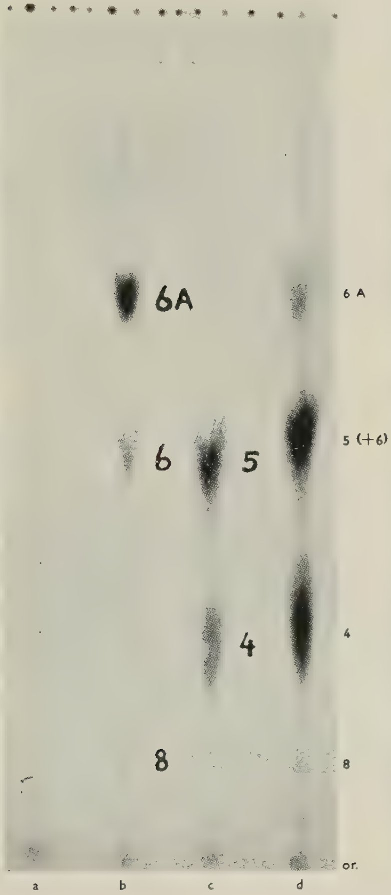


Fig. 2

Fig. 3

ACTION OF THYROID PROTEASE ON THYROGLOBULIN

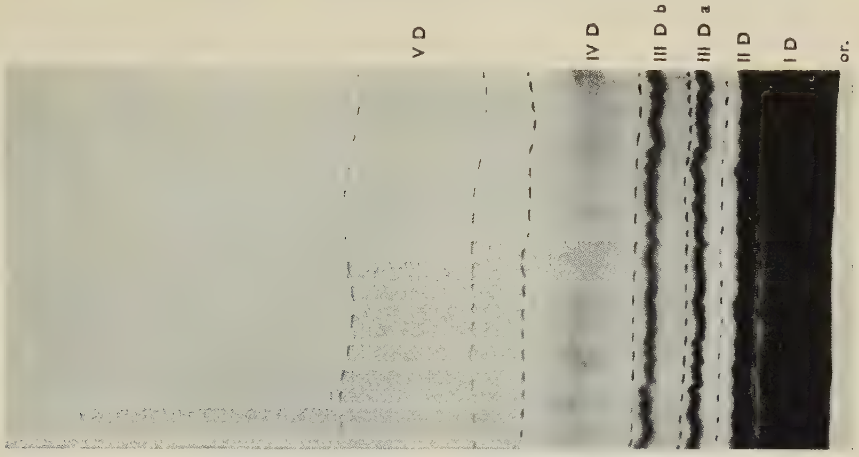


Fig. 6

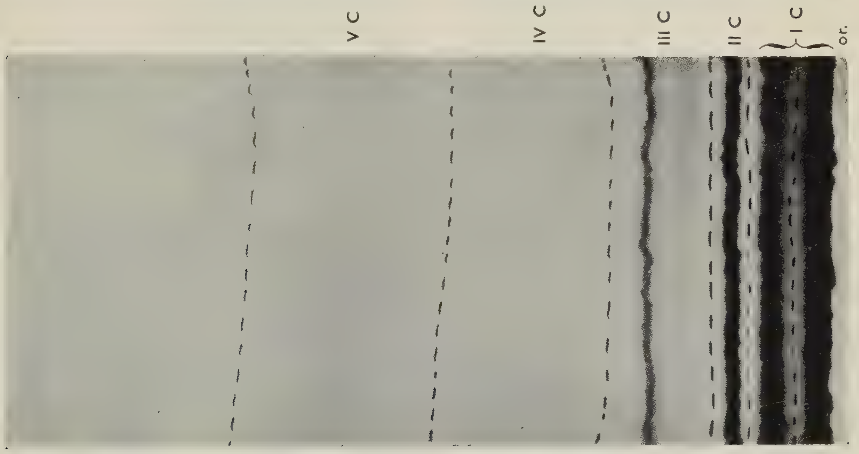


Fig. 5

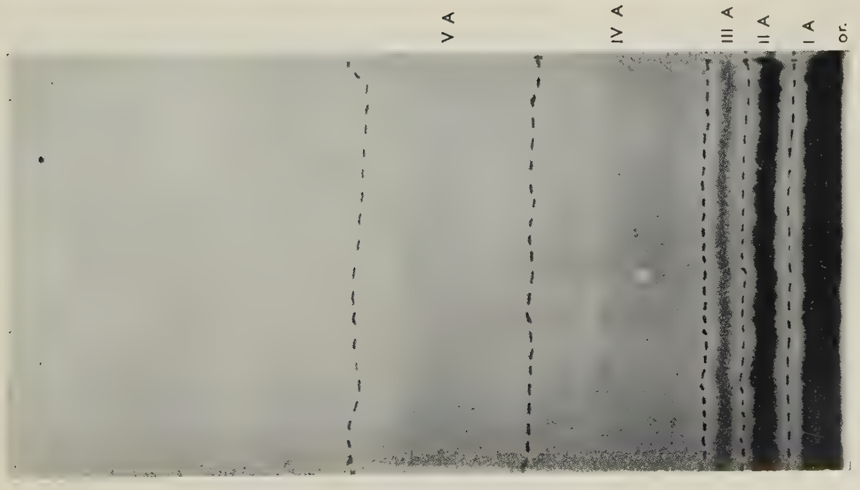
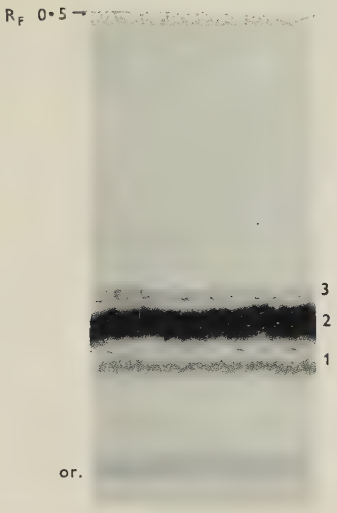


Fig. 4

ACTION OF THYROID PROTEASE ON THYROGLOBULIN



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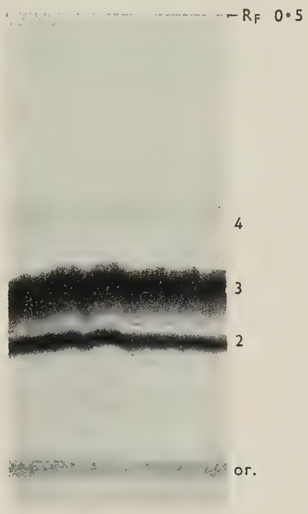

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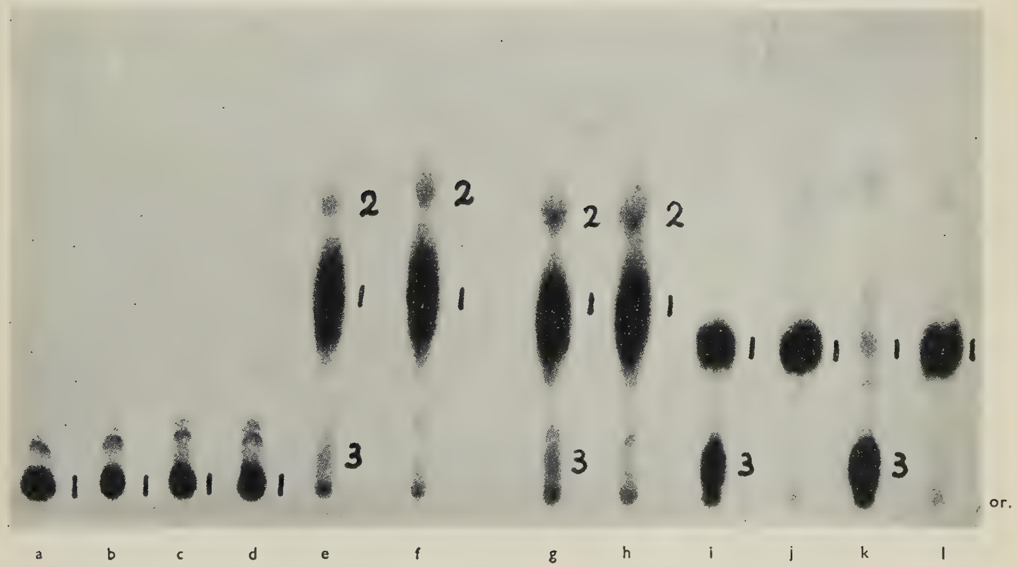

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of di-iodo-APAT by pepsin was almost complete compared with about 50 per cent. by the thyroid protease. By contrast only trace amounts of di-iodotyrosine were liberated by either enzyme from the leucyl peptide. The iodinated glycyl peptide was found to have an R_F value very close to di-iodotyrosine in all the solvent systems tried. However, inspection of Plate 3, Figure 9, will show a very close similarity between the four radioautograms corresponding to the digests of the peptide with both heated and unheated enzymes. Any appreciable release of di-iodotyrosine would have led to an elongation of the peptide spots. The significance of these findings will be discussed later.

Table 6

DESIGN OF EXPERIMENT TO COMPARE THE ACTION OF THYROID PROTEASE AND PEPSIN ON LABELLED IODOPEPTIDES (pH = 3·2)

Tube No.	Peptide*	Thyroid Protease (ml)	Pepsin (ml)
1	Glycyl-	0·05	—
2	Glycyl-	0·05 (HI)†	—
3	Glycyl-	—	0·05 (HI)
4	Glycyl-	—	0·05
5	Leucyl-	0·05	—
6	Leucyl-	0·05 (HI)	—
7	Leucyl-	—	0·05
8	Leucyl-	—	0·05 (HI)
9	Acetyl PA-	0·05	—
10	Acetyl PA-	0·05 (HI)	—
11	Acetyl PA-	—	0·05
12	Acetyl PA-	—	0·05 (HI)

*Glycyl-, leucyl- and acetyl PA- = the iodinated derivatives of glycyl-L-tyrosine, L-leucyl-L-tyrosine and *N*-acetyl-L-phenylalanyl-L-tyrosine respectively. In all cases volume of peptide solution = 0·1 ml.

† HI = Heat-inactivated.

V. Discussion

The results of seven preparative experiments recorded in this paper (Table 1) show that it is possible to obtain rapidly by the fractional precipitation method of Derrien, Michel, and Roche (1948) purified labelled thyroglobulin in which 50 per cent. or more of the ^{131}I taken up by the animals' thyroids is retained by this fraction. The dry weight of the protein recovered amounted to approximately 5 per cent. of that of the dissected thyroid tissue in five out of seven of the experiments. With such preparations as substrates it has been demonstrated that digestion with purified thyroid protease can lead to the release, after 24 hr at 37°C and pH 3·5, of nearly 40 per cent. of the radioactivity in the form of compounds now soluble in 70 per cent. ethanol. This was considerably greater than the degradation which followed incubation at 0°C or that obtained when the endogenous protease in the thyroglobulin was allowed to act at 37°C in the presence of heat-inactivated added enzyme. From Table 3 (Experiment 2) it is seen that the percentage distribution of radioactivity in the several chromatographic zones is substantially similar for the digest at 37°C and for the two controls. It therefore seems likely that the added protease is closely related to, if not identical with, the enzyme retained by the thyroglobulin during its preparation.

Following resolution of the alcoholic extracts of the enzymic digests by paper chromatographic procedures, 3-mono-iodotyrosine (MIT), 3,5-di-iodotyrosine (DIT), thyroxine (Tx), 3,5,3′-tri-iodothyronine (TIT), and a "peptide" fraction have been demonstrated as the major radioactive iodine-containing components present. Mono-iodohistidine (characterized by Roche, Lissitzky, and Michel (1952) as a component of thyroglobulin) was also identified in trace amounts, while the nature of another radioactive spot of moderate intensity, in a position on the chromatogram between iodide and Tx (*n*-butanol-*n*-pentanol-2N NH_4OH system), could not be determined. Both these substances were also present in the alkaline hydrolysates of Experiment 3 (Plate 3, Fig. 7). After allowing for the lower content of Tx and TIT in thyroglobulin compared with DIT and MIT the former two iodoamino acids do not appear to be released preferentially by the enzyme (cf. Michel 1952).

The nature of the "peptide" fraction was not ascertained. The increase, greater than threefold (2·02 μc compared to 0·59 μc) in the amount of "peptide," corresponding to the increase in incubation temperature from 0° to 37°C in Experiment 2, suggests, as more likely, that this fraction is a product of enzyme action rather than a pre-existing loosely attached component of the thyroglobulin dissociable in 70 per cent. ethanol. Furthermore, since the activity ratios of DIT to "peptide" observed in zone I of both Experiments 2 and 3 were found to be approximately equivalent (Table 3), this could be used as an argument against contamination by thyroglobulin itself; it is, however, true that the specific activity values of the "peptide" found in both experiments were close to those of the corresponding thyroglobulins (Table 5).

The decreasing trend in the order of specific activities, namely MIT > DIT > Tx, in the alkaline hydrolysate of Experiment 3 (Table 5) demonstrates the generally accepted sequence of these amino acids during their biosynthesis as MIT → DIT → Tx (cf. Taurog and Chaikoff 1947). However, this order does not apply to the specific activities of the labelled components of the corresponding enzyme digest (Table 5). Furthermore, the individual values for MIT and DIT liberated by enzyme action are appreciably different from those obtained following alkaline hydrolysis. These discrepancies could have risen from errors in technique although all procedures were standardized and estimations of ^{127}I made in duplicate in most instances. For example, even though our laboratory has had considerable experience in the micro-estimation of iodine, such procedures as elution and evaporation, associated with the preparation of the purified zonal components, may have introduced contamination with ^{127}I. All chromatographic paper was, however, subjected to prolonged washing with distilled water before use. It should also be noted that the specific activities of the DIT and "peptide" fractions in E_0 (Experiment 2) were determined although the values are not included in Table 5; these were 0·11 and 0·082 μc $^{131}I/\mu g$ ^{127}I respectively, which are close to the values obtained for the same two components in E_{37}. Furthermore, replicate determinations of the specific activities of the thyroglobulin used in Experiment 3 gave the following values: 0·072, 0·080, 0·077, 0·074 μc $^{131}I/\mu g$ ^{127}I.

If the observed variation in the specific activity values between the corresponding components of the enzymic and alkaline hydrolysates is accepted as significant, this might indicate a non-homogeneous biosynthesis of thyroglobulin. This suggestion lacks, however, the evidence which could have been provided by determinations of the specific activities of the components of the fraction insoluble in 70 per cent. ethanol; these were, unfortunately, not carried out.

The action of thyroid protease on the labelled peptides (within the optimal pH range of the enzyme for protein (haemoglobin)) suggests that this enzyme has properties similar to those found for pepsin by Baker (1951), namely that it hydrolyses preferentially links between two aryl-amino acids. Although we have no evidence for a preferential release by the enzyme of thyroxine from thyroglobulin, compared with the other iodoamino acids, it might prove to be the case that the release of *all* iodoamino acids is favoured during proteolysis. This could be brought about by the presence, adjacent to the iodoamino acids (all of which contain aromatic residues), of other arylamino acids. Weiss (1953) has recently effected a partial separation of the peptidase and protease activities of thyroid tissue (tested respectively at pH 7·8 and pH 4·0). However, he does not appear to have examined his protease fraction for peptidase activity at acid pH.

VI. Acknowledgments

One of us (M. T. McQ.) is indebted to the Australian National Health and Medical Research Council for a personal grant and for financial assistance towards the cost of the investigation. We also wish to thank Mrs. D. Winikoff for the iodine assays. Dr. C. E. Eddy, Director, Commonwealth X-Ray and Radium Laboratory, kindly provided facilities for the radioautography.

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EXPLANATION OF PLATES 1-3

PLATE 1

Fig. 1.—Radioautogram of chromatogram of samples of zones *M* and *L* eluted from the PWA (phenol-water-ammonia) chromatograms of the products of thyroid protease action. Experiment 1. Compare with Figures 2 and 3 but note that more material applied in preparing chromatograms in latter cases. (*a*) Zone *M* eluate. Solvent system, BPA (*n*-butanol-*n*-pentanol-ammonia); (*b*) zone *L* eluate. Solvent system, BPA. 1, "Peptide"; 2, di-iodotyrosine; 3, mono-iodotyrosine; 4, thyroxine; 5, tri-iodothyronine; *or.*, origin.

Fig. 2.—Radioautogram of chromatogram of *n*-butanol extracts of eluates of zones *L* and *M* after treatment with alkali at 0° and 105°C, respectively, for 16 hr. Butanol extract at pH 14 (2N NaOH). (*a*) Zone *L* eluate, 0°C; (*b*) zone *L* eluate, 105°C; (*c*) zone *M* eluate, 0°C; (*d*) zone *M* eluate, 105°C. Solvent system, BPA. 2-5 As for Figure 1; 6, alkali-produced artefact not identical with but close to 5; 6*A*, alkali-produced artefact; 8, iodide; *or.*, origin.

Fig. 3.—Radioautogram of chromatogram of *n*-butanol extracts of eluates of zones *L* and *M* after treatment with alkali at 0° and 105°C, respectively, for 16 hr. Butanol extract prepared after adjustment to pH 3·5 of residue from extraction at pH 14 (Fig. 2). (*a*) Zone *L* eluate, 0°C; (*b*) zone *L* eluate, 105°C; (*c*) zone *M* eluate, 0°C; (*d*) zone *M* eluate, 105°C. Solvent system, BPA. 1-6 As for Figures 1 and 2; 7, unknown; 7*A*, unknown, R_F as 4 but identity with thyroxine disproved; 8, iodide; *or.*, origin.

PLATE 2

Fig. 4.—Radioautogram of chromatogram of the 70 per cent. ethanol-soluble fraction of E_{37} enzyme digest. Experiment 2. Solvent system, BPA. The zones indicated refer to those in Table 3. *or.*, origin.

Fig. 5.—Radioautogram of chromatogram of the 70 per cent. ethanol-soluble fraction of E_{37} enzyme digest. Experiment 3. Solvent system, BPA. The zones indicated refer to those in Table 3. *or.*, origin.

Fig. 6.—Radioautogram of chromatogram of the *n*-butanol extract prepared at pH 3·5 from the alkaline hydrolysate of labelled thyroglobulin. Experiment 3. Solvent system, BPA. The zones indicated refer to those in Table 4. *or.*, origin.

PLATE 3

Fig. 7.—Radioautogram of chromatogram of zone III*Da* eluate after re-running in the solvent system BPA. 1, Mono-iodohistidine; 2, iodide; 3, unknown; 4, thyroxine; *or.*, origin.

Fig. 8.—Radioautogram of chromatogram of zone III*Db* eluate after re-running in the solvent system BPA. 2, Iodide; 3, unknown; 4, thyroxine; *or.*, origin.

Fig. 9.—Radioautogram of chromatograms of three labelled dipeptides containing di-iodotyrosine after incubation with active and heat-inactivated (HI) enzymes. Solvent system, BPA. (*a*) Glycyl— + thyroid protease; (*b*) glycyl— + thyroid protease (HI); (*c*) glycyl— + pepsin (HI); (*d*) glycyl— + pepsin; (*e*) L-leucyl— + thyroid protease; (*f*) L-leucyl— + thyroid protease (HI); (*g*) L-leucyl— + pepsin; (*h*) L-leucyl— + pepsin (HI); (*i*) *N*-acetyl-L-phenylalanyl— + thyroid protease; (*j*) *N*-acetyl-L-phenylalanyl— + thyroid protease (HI); (*k*) *N*-acetyl-L-phenylalanyl— + pepsin; (*l*) *N*-acetyl-L-phenylalanyl— + pepsin (HI). 1, Unchanged peptide; 2, iodide; 3, di-iodotyrosine; *or.*, origin.

THE ELASTIC ANISOTROPY OF KERATINOUS SOLIDS

I. THE DILATATIONAL ELASTIC CONSTANTS

By K. Rachel Makinson*

[*Manuscript received February* 17, 1954]

Summary

The elastic anisotropy of four forms of α-keratin, ram's horn, rhinoceros horn, baleen (whalebone), and the cortex of African porcupine quill, has been studied by measurement of the velocity of propagation of dilatational elastic waves of 5 Mc/s frequency along the principal axes, by the total reflection method. It has been found that ram's horn is transversely isotropic about the radial direction and that rhinoceros horn is approximately transversely isotropic about the fibre direction. This is directly correlated with the histological structure of these materials, which here predominates over the molecular structure in determining the nature of the elastic anisotropy.

All three principal velocities and the corresponding elastic constants have been determined for ram's horn and rhinoceros horn, and two of them for baleen. Some less accurate data are presented for two of the principal directions in African porcupine quill cortex. For ram's horn the approximate values of the elastic constants which govern the propagation of dilatational waves along and perpendicular to the radial direction are $0 \cdot 85$ and $1 \cdot 01 \times 10^{11}$ dyn cm^{-2} respectively; for rhinoceros horn perpendicular and parallel to the direction of growth, $0 \cdot 84$ and $1 \cdot 09 \times 10^{11}$ dyn cm^{-2}.

I. Introduction

Owing to the complexity of the molecular structure of keratin and to the inhomogeneity of the available keratinous materials, the molecular structure cannot be determined completely from X-ray diffraction data alone. Other data have also to be considered; among these, the viscoelastic properties are important.

Except in a few cases (e.g. Warburton 1948; MacArthur 1943) the elastic properties of α-keratin have been studied only for keratin in the form of fibres; the obvious limitations of both the interpretation and the technique of transverse measurements have confined such studies to extension or contraction along the fibre axis and torsion about this axis. The high extensibility along the axis has been used by Astbury and many later workers as a basis for the postulation of the various folded chain structures of α-keratin. Fruitful as this concept has been, the validity, in general, of the direct attribution to the molecule of the total elastic behaviour of an anisotropic, inhomogeneous keratin fibre, with its well-defined histological structure, cannot be regarded as firmly established. If a complete study were made of the anisotropy of various forms

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of keratin over a wide range of strains and frequencies, it might be possible to separate that component of the elastic behaviour which is due to the molecules of crystalline keratin from those components which are due to the histological structure and to the disordered regions. In the range of very small strains, determination of all the principal elastic constants of different forms of keratin might provide data which could be used as a test of proposed molecular structures. Even an incomplete study could test the relative importance of the molecular and the histological structures in determining the elastic behaviour.

The data obtained would be useful in other problems also, such as that of the absorption of water by keratin (cf. Cassie 1945).

A complete study will obviously be very lengthy, and will have to be carried out gradually. A beginning has been made in the work to be described in this and a subsequent paper.

II. Experimental Method

The experimental technique used has been described elsewhere (Makinson 1952) in its application to isotropic solids, and will only be outlined here. A thin, acute-angled wedge of the solid is immersed vertically in oil in the path of a horizontal ultrasonic beam of frequency 5 Mc/s. The beam transmitted through the wedge is observed by the diffraction which it produces in a perpendicular beam of light. The angle of incidence ϕ' is measured at which certain characteristic zeros of transmission occur for several different regions along the wedge. This angle is the critical angle of total reflection of the dilatational (or longitudinal) elastic wave, which is then propagating in the solid parallel to the width of the wedge, i.e. $\phi' = \sin^{-1} \omega_d/\omega_0$, where ω_0 is the velocity of the ultrasonic wave in the oil and ω_d is the velocity of the dilatational wave in the solid. Hence ω_d is calculated. The corresponding elastic constant is equal to $\rho\omega_d^2$, where ρ is the density of the solid. It is greater than the Young's modulus of the solid (see Section VI).

The ultrasonic power used is very low, so that all strains in the solid are very small and lie in the elastic region.

The method is not in general applicable to an anisotropic solid, since in this case there is in general no purely dilatational refracted wave. It is applicable if (i) the crystallographic axes of the solid are mutually perpendicular, and (ii) the principal directions of the wedge lie along these axes. The method was applicable, either exactly or to a close approximation, to all the specimens studied in the main line of this work.

Following usual crystallographic practice, as summarized by Hearmon (1946), the dilatational elastic constant in the i direction is written c_{ii}, e.g. the elastic constants governing the propagation of dilatational waves in the r, θ, and z directions are written c_{rr}, $c_{\theta\theta}$, and c_{zz}.

There are practical difficulties in applying the method to keratinous solids, since the available specimens of keratin are inhomogeneous, of inconvenient shapes, and of limited sizes. It was not always possible to cut sufficiently large and well-oriented wedges in all the directions necessary for the determination of all three of the constants c_{ii}, so that some of the data are incomplete. Nevertheless, it was possible to draw some unambiguous conclusions from the measurements.

In the cortex of ram's horn regions of slightly different densities were found to occur. The density was determined with respect to the oil which surrounded the specimens. The values for the wedges used fell into two groups, around 1·28 and 1·31 g cm^{-3}, the difference being greater than the experimental error. The grouping did not correspond to particular orientations of the wedges, so that the lower densities are unlikely to have been due to such effects as penetration of air along the grain. It was impossible to avoid altogether the use of the material of lower density. However, the results obtained showed no correlation between this density variation and the measured velocity, whereas there was complete correlation between the direction of propagation and the velocity. In calculation of the elastic moduli c_{ii} for horn, the actual density relative to the oil for each specimen has been used, but only about 2 per cent. change would have been made in any value of the moduli by using a mean value of the density, and this would not have affected the main conclusions.

A less thorough study of density variations was made for the other materials used. The range for rhinoceros horn was 1·29 to 1·31, the value for the outer layer of baleen was 1·29, and that for the side wall of porcupine quill, after it had been boiled under moderate pressure to flatten it, was 1·32.

The regain of the keratin was not closely controlled, nor was the temperature of the measurements. It has been found by other workers (Chaikin 1953) and was confirmed by the results described here, that at frequencies in the megacycle range the elastic constants are much less sensitive to regain and temperature than at low frequencies. The specimens were stored and used in a room in which the day temperature varied from about 17·5° to about 22°C, being usually near 20°C, and the day humidity varied from about 35 to 60 per cent. For measurement, the specimens were immersed in a 4-gal tank of technical white oil which over a period of a year or more had come to equilibrium with the average moisture content of the air in the room. No dependence of the measurements on the room conditions was observed.

III. Preparation of the Specimens

The materials used were ram's horn, rhinoceros horn, and baleen (whalebone) from a rorqual. A few data which were of only qualitative value were obtained with African porcupine quill. In general, plates as large as possible up to about 8 by 4 by 0·5 cm were cut at the required orientation from the cortex of the various materials, and were formed into wedges of from 0·12 to 2·0 mm thickness by cementing each side in turn to a steel template with celluloid cement and shaping them with a shaper (milling and sanding were found to be unsatisfactory techniques). The wedges were then released from the template with amyl acetate and washed in the same liquid. For the baleen the outer layer, or "cut," was used. For porcupine quill (*a*) plates were made by cementing together a number of pieces of tip; (*b*) cylindrical pieces of quill cortex were flattened out in boiling water, as described by Rudall (1936). This treatment, of course, may have changed the structure of the quill. Neither process gave satisfactory specimens, and the data obtained are of only qualitative value.

The nomenclature adopted for the crystallographic axes, in the ram's horn, baleen, and porcupine quill, was: z axis, the direction of growth, i.e. the "fibre" direction; r axis, the radial or thickness direction; θ axis, the circumferential or width direction. These were rectilinear Cartesian, not polar axes, since the ram's horn had an approximately triangular section, from which flat wedges could be cut, the baleen was naturally flat, and the porcupine quill was used flattened out. In rhinoceros horn the principal directions were taken as: z axis, direction of growth (fibre direction); l axis, direction of major (long) diameter of cross section; s axis, direction of minor (short) diameter of cross section.

The relation of these directions to the structure of the materials will be made clearer in Section V.

Owing to the spiral growth of ram's horn, the direction of the z axis varied along the wedge. The straightest portions were used as far as possible, and the misalignment was usually not greater than 4-5° at the important parts of the wedges. It was found that the measured velocity was not very sensitive to this error, owing partly to the low anisotropy of ram's horn.

IV. Experimental Results

The values of the elastic constants are shown in Figure 1 and Table 1, and the wave velocities are also given in Table 1. Some comments are necessary on the estimation of the errors. The specimens I to XI of ram's horn were all different wedges, and for any set of specimens in which the width of the wedge, i.e. the direction of propagation of the dilatational wave, lay along one crystallographic axis (e.g. r), some of the specimens had their length along the second crystallographic axis (e.g. θ) and others had their length along the third axis (e.g. z). For each of the specimens except V, VIII, X, and XI the wedge was long enough to permit the observation of two series of transmission zeros (these were not always the first and second series described previously (Makinson 1952)). The two values for XI both refer to the same series. As has been shown previously (Makinson 1952) there is a small systematic discrepancy between the values of ϕ' for different series of zeros. Also, the distribution of points in any one series is not usually Gaussian but shows a definite trend with thickness of the wedge, and for some orientations of the crystallographic axes the zeros are wider than for others. Generous estimates have therefore been made of the errors for each series of zeros by taking as "likely error" that corresponding to the extreme angular range of the centres of the zeros in each series, and as "extreme possible error" that corresponding to the extreme angular range over which zeros of transmission extend. This latter almost certainly considerably over-estimates the error for any one set of measurements.

The specimens of rhinoceros horn sub-indexed (*b*) and (*c*) were made from the corresponding (*a*) by machining it thinner. This shifted the positions of the minima along the wedges, so that a different part could be studied. These wedges were longer than those of ram's horn, so that it was sometimes possible to obtain three series of zeros. All the values given for any one specimen refer to different series.

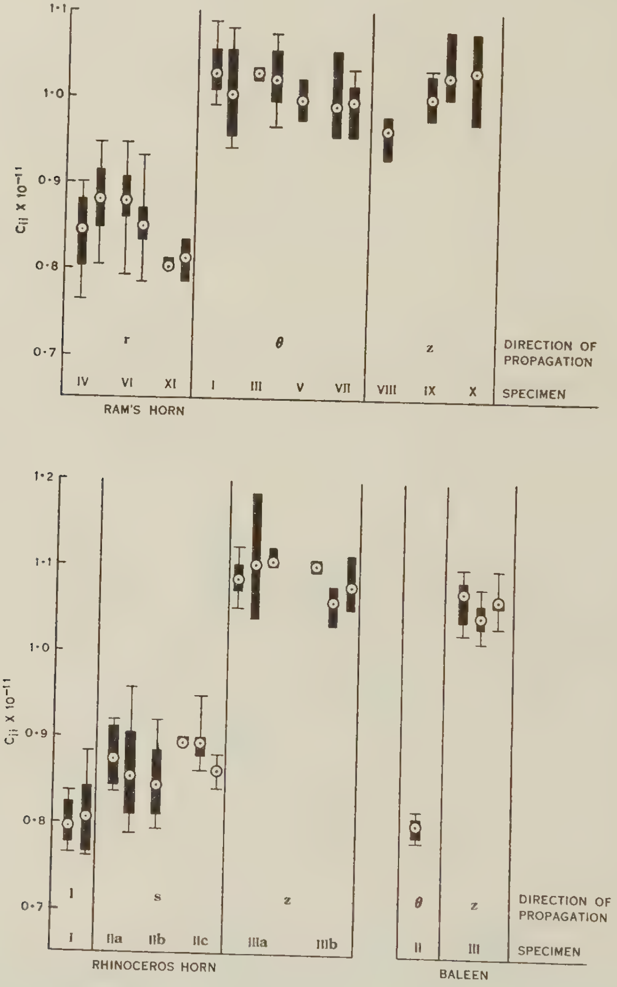

Fig. 1.—Dilatational elastic constants c_{ii} of some forms of α-keratin: Broad line indicates "likely error" and thin line "extreme possible error" (see text).

It must be remembered that there will also be variations in the measured moduli arising from true variability of the keratinous materials.

The data of Table 1 and Figure 1 show that ram's horn is transversely isotropic about *r* (i.e. the elastic behaviour is the same in all directions perpendicular to r), whereas rhinoceros horn is approximately transversely isotropic about z; rhinoceros horn may in fact be orthorhombic, but the difference between the l and s directions is barely significant. For baleen and for porcupine quill, owing to their thinness in the r direction, data could be obtained only in the z and θ directions.

That the ram's horn was transversely isotropic about r, and not, for example, tetragonal, was confirmed by measurements on two wedges cut in the θz plane with their lengths at about 30° to z. These measurements were made later, under less favourable conditions, and were less accurate. However, they gave as the value of the dilatational velocity in the θz plane, at 60° to z, about $2{\cdot}7$ or $2{\cdot}8 \times 10^5$ cm/sec, as for propagation parallel to θ or z.

Measurements were also made on a wedge cut with its length parallel to z and its faces at 45° to r and θ. Propagation in this case was poor; the velocity was in the neighbourhood of $2{\cdot}5$ or $2{\cdot}6 \times 10^5$ cm/sec and was definitely less than $2{\cdot}7 \times 10^5$. Here the orientation was such that the method was not strictly applicable, and the result is only of qualitative value.

The transverse isotropy of ram's horn about r was further confirmed by independent measurements of the velocity of propagation of a dilatational pulse along each of the crystallographic axes of specimens cut in the form of small slabs a few millimetres thick. The frequency was 4 Mc/s. The author was enabled to make these measurements by the kindness of Dr. G. Bradfield, of the National Physical Laboratory (U.K.), who permitted the use of his apparatus, a modified form of that described previously (Bradfield 1950). The absolute values so determined are not directly comparable with those of Figure 1 and Table 1, since in the N.P.L. method it is necessary to apply considerable static pressure to the specimens, whereas none is applied in the total reflection method. However, it is clear that within the accuracy of the measurements $c_{\theta\theta} = c_{zz} > c_{rr}$, as found by the total reflection method.

V. Correlation of Elastic Anisotropy with Structure

The most complete of the elastic data obtained are those for ram's horn and rhinoceros horn. The histological structure of each is shown diagrammatically in Figure 2. The cortex of ram's horn is composed of flat cells lying with their faces perpendicular to r. When a thin section cut in any direction was treated with concentrated sulphuric acid the cells separated immediately into layers perpendicular to r, but their separation into individual cells was much slower. It is therefore clear that histologically the structure is approximately transversely isotropic about r.

According to Rudall (1936), when keratin occurs in the form of flat cells the fibrils run in the plane of the cell either in a dispersed manner about z or else predominantly in two directions inclined at equal and opposite angles to z. Rudall has found that in cow's horn this angle is about 30°. For cow's horn the X-ray diffraction data (Astbury and Sisson 1935; Rudall 1936) show an α-keratin structure poorly oriented along z, with complete transverse isotropy about z. Rudall considers this compatible with the generally accepted view

Table 1

DILATATIONAL ELASTIC CONSTANTS c_{ii} AND DILATATIONAL WAVE VELOCITIES ω_d FOR SOME FORMS OF α-KERATIN

Material	Direction of Propagation	Specimen	$\omega_d \times 10^{-5}$ (cm sec^{-1})			c_{ii}† $\times 10^{-11}$ (dyn cm^{-2})
			Mean	Likely* Range	Extreme* Range	
Ram's horn	r	IV	2·53	2·58–2·47	2·61–2·41	0·844
			2·58	2·63–2·53	2·68–2·47	0·880
		VI	2·63	2·67–2·60	2·73–2·49	0·879
			2·58	2·61–2·55	2·70–2·48	0·850
		XI	2·51	2·53–2·51	2·53–2·51	0·802
			2·53	2·56–2·49	2·56–2·49	0·812
	θ	I	2·80	2·83–2·78	2·88–2·75	1·029
			2·77	2·83–2·70	2·87–2·68	1·004
		III	2·80	2·81–2·79	2·81–2·79	1·029
			2·79	2·84–2·76	2·87–2·72	1·022
		V	2·76	2·79–2·73	2·79–2·73	0·998
		VII	2·76	2·85–2·71	2·85–2·71	0·990
			2·77	2·79–2·71	2·82–2·71	0·996
	z	VIII	2·71	2·74–2·67	2·74–2·67	0·964
		IX	2·78	2·81–2·74	2·82–2·74	1·002
			2·81	2·88–2·78	2·88–2·78	1·027
		X	2·79	2·85–2·70	2·85–2·70	1·031
Ram's horn; pulse measurements	r	3	2·71	2·68–2·73		0·959
		4	2·65	Small		0·922
	θ	1	2·85	2·82–2·88		1·057
		2	2·84	2·78–2·90		1·050
	z	5	2·89	Small		1·070
		6	2·86	Small		1·048
Rhinoceros horn	l	I	2·47	2·51–2·44	2·53–2·42	0·797
			2·48	2·53–2·42	2·60–2·41	0·806
	s	II*a*	2·60	2·66–2·56	2·67–2·55	0·875
			2·60	2·65–2·51	2·73–2·47	0·855
		II*b*	2·56	2·62–2·51	2·67–2·48	0·846
		II*c*	2·63	2·64–2·63	2·64–2·63	0·894
			2·63	2·64–2·61	2·71–2·58	0·894
			2·58	Small	2·61–2·55	0·861
	z	III*a*	2·90	2·92–2·88	2·95–2·86	1·086
			2·92	3·03–2·84	3·03–2·84	1·101
			2·93	2·95–2·92	2·95–2·92	1·107
		III*b*	2·92	2·93–2·91	2·93–2·91	1·101
			2·87	2·89–2·83	2·89–2·83	1·059
			2·89	2·94–2·86	2·94–2·86	1·078
Baleen (whale-bone) from a rorqual	θ	II	2·49	2·51–2·47	2·52–2·46	0·802
	z	III	2·88	2·90–2·84	2·92–2·82	1·072
			2·85	2·87–2·83	2·89–2·80	1·046
			2·87	Small	2·92–2·83	1·065

Table 1 (*Continued*)

African porcupine quill cortex, boiled side wall	θ z	Several Several	≃2·6 ≃3·1	2·7–2·4 3·2–3·0	Unknown Unknown	≃0·9 ≃1·3
Same, tip, unboiled	θ z	Two specimens Two specimens	≃2·9 ≃3·4	3·0–2·7 3·5–3·3	Unknown Unknown	≃1·1 ≃1·5

*See text for definition.

†The ranges of c_{ii} are shown in Figure 1.

that the chains of the α-keratin structure lie along the fibrils, since the dispersion of the fibril directions is too great to permit the diffraction pattern to be resolved into two patterns crossed at 30°. The structure of ram's horn is very similar, except that the fibrils appear to be dispersed or crossed about z at angles up to about 20° from z. No orientation of the side-chain and backbone directions in the plane normal to z has been detected for any form of α-keratin. Molecularly, therefore, ram's horn is poorly oriented, but its average structure is one of transverse isotropy about z.

The data given here for ram's horn therefore show that the elastic anisotropy is predominantly determined by the histological rather than by the molecular structure. This illustrates the invalidity in general of direct argument from the elastic properties to the molecular structure in histologically complex materials.

Rhinoceros horn is also formed of flat cells, but they are arranged in concentric layers in component cylindrical structures which frequently are medullated (Fig. 2). These structures, which are tightly packed together, are of the order of 300-500 μ in diameter. The medullae, which are frequently flattened into a dumb-bell shape, do not show any marked preferential orientation with respect to the l and s axes. When thin sections were treated with concentrated sulphuric acid, there was instantaneous swelling and the cells peeled off in layers concentric with the cylinders. Over a macroscopic volume of rhinoceros horn, therefore, the histological structure is transversely isotropic about z. The molecular structure, as in horn, would be expected to be transversely isotropic about z, so that the histological and the molecular anisotropy are similar and should result, as is in fact observed, in an elastic anisotropy of the same kind.

The structure of baleen (whalebone) varies across the thickness (r) as shown in Figure 2 (Fraser 1952). The portion which was used for this study was the smooth outer layer, known as the "cut," in which the cells are flat and lie perpendicular to r. Wedges were prepared by removing the fibres from the inner side with a shaper. As the maximum thickness of the cut was about 1·5 mm, no wedges could be made with their width parallel to r, so that c_{rr} could not be measured. The wedges so prepared still showed a coarse striation of the same order of size as the fibres, and microscopic examination showed that on the (formerly) inner surface the cell layering was curved in the region which had been in contact with the fibres. On treatment with concentrated sulphuric acid the cells peeled off in flat layers perpendicular to r, but it was noticeable that there was a considerable difference between the θ and z directions. Swelling was greater parallel to θ than to z, and the layers split more easily parallel to z than to θ. In some cells the fibrils ran predominantly along the z direction, in others they were crossed, and frequently they were invisible.

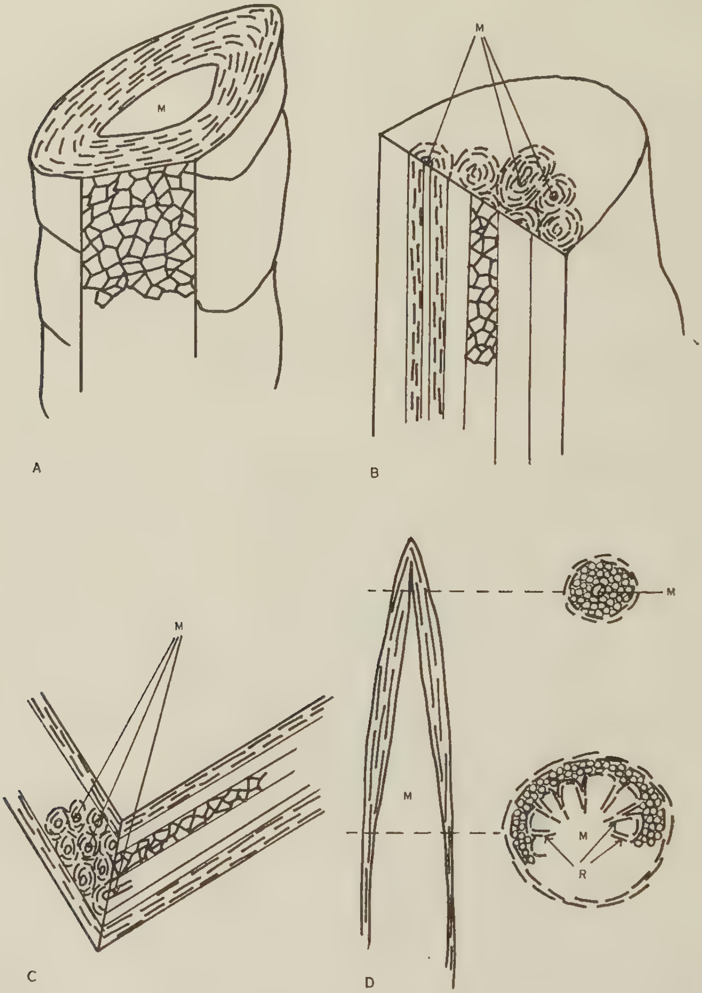

Fig. 2.—Histological structure of some forms of α-keratin: purely diagrammatic and not to scale. *a*, Ram's horn; *b*, rhinoceros horn; *c*, baleen (whalebone); *d*, African porcupine quill. *M* denotes the medullae and *R* the rays (see text).

This structure is correlated with the measured elastic constants, which show $c_{zz} > c_{\theta\theta}$. As explained above, c_{rr} could not be measured.

The cortex of African porcupine quill also has a complicated structure (Rudall 1936). At the tip it consists (see Fig. 2) of spindle-shaped cells lying parallel to z; further down the quill it consists of three layers, a thin layer of flat cells enclosing spindle-shaped cells, inside which there are again flat cells surrounding the medulla and forming the rays. Most of these inner flat cells were removed in the preparation of the specimens. On treatment with concentrated sulphuric acid the cells tended to break loose in blocks between the roots of the rays. As a secondary stage they peeled off into flat sheets with marked striation parallel to z and occasional striation perpendicular to this. These sheets were predominantly parallel to θz, but curved round towards the rz plane near the edges of the blocks. At a still later stage the sheets broke up parallel to z into spindle-shaped cells. The structure may be summarized as a sandwich of spindle-shaped cells between flat cells, all three layers being composed of sheets which lie predominantly parallel to θz, but with a tendency to form cylindrical blocks between the roots of the rays.

Histologically, therefore, the structure of the tip is transversely isotropic about z, and that of the opened-out side wall is orthorhombic. In spindle-shaped cells, according to Rudall, the fibrils, along which lie the molecular chains, run along the z axis and there is again no preferential orientation of the side-chain and backbone directions in the $r\theta$ plane, so that molecularly both the tip and, to a first approximation, the side wall should be transversely isotropic about z. This has been confirmed for the tip by the X-ray diffraction studies of MacArthur (1943). The few elastic data obtained (see Table 1) are of very low accuracy and show only that $c_{zz} > c_{\theta\theta}$ and that the degree of anisotropy is quite high. This conclusion applies also to the specimens boiled under pressure, although their structure may have been changed by the treatment.

VI. Relation to Results Obtained by Other Workers

The dilatational moduli c_{ii} have not previously been measured for any form of α-keratin. Measurements have been made of some of the Young's moduli, which for isotropic solids with Poisson's ratio 0·25 would be about 17 per cent. less than the dilatational moduli measured under the same conditions.

The only measurements yet published on any of the materials used here are those by Warburton (1948), who measured the Young's modulus of sheep's horn in the θ direction (E_θ) as a function of regain, at low audio frequencies. He found that at 12 per cent. regain (which corresponds to a relative humidity in the neighbourhood of 50 per cent.) E_θ was approx. $0{\cdot}5 \times 10^{11}$ dyn cm^{-2}. This is much lower than the value, about $1{\cdot}0 \times 10^{11}$ dyn cm^{-2}, reported here for the dilatational modulus, as is to be expected, since all the available evidence shows that the moduli increase with frequency, at least in the range from 0 to 100 kc/s (Chaikin 1953). This increase is presumably due to the blocking of the slower relaxation processes.

Chaikin (1953) quoted Terry and Woods (unpublished data) as having measured the velocity corresponding to the Young's modulus in the z direction of "horn" at 50 kc/s. The value they obtained was $2{\cdot}0_5$-$2{\cdot}2_3 \times 10^5$ cm sec^{-1}, which corresponds to values of E_z from $0{\cdot}55$ to $0{\cdot}65 \times 10^{11}$ dyn cm^{-2}. The humidity was not specified. In a personal communication, Dr. Terry has given the mean velocity parallel to z in cow's horn at uncontrolled room humidity as

$2{\cdot}15 \times 10^5$ cm/sec^{-1}, which with $\rho = 1{\cdot}3$ g cm^{-3} gives $E_z = 0{\cdot}60 \times 10^{11}$ dyn cm^{-2}. These values lie between Warburton's low frequency value for E_θ and the high frequency (5 Mc/s) value obtained here for the dilatational modulus parallel to either θ or z.

There have been numerous determinations of the longitudinal Young's moduli E_z of keratin fibres; these have been surveyed by Chaikin (1953). The highest frequency used was that which he used himself, 102·5 kc/s. At 65 per cent. relative humidity and 22·2°C he found that for human hair the longitudinal Young's modulus E_z was about $0{\cdot}84 \times 10^{11}$ dyn cm^{-2}, increasing slightly as the humidity decreased. The value for Lincoln wool was of the same order. If the Poisson's ratios of the forms of keratin considered are close to 0·25, this value of E_z is close to those which could be deduced from the dilatational constants c_{zz} of ram's and rhinoceros horn at 5 Mc/s by treating the materials as approximately isotropic. It is possible, therefore, that dispersion is small (relaxation processes becoming less important) between 100 kc/s and 5 Mc/s, but this cannot be regarded as established until further data are available.

VII. Conclusions

The most important conclusion to be drawn from this study is the qualitative one that the elastic anisotropy of keratinous solids in the region of small strains depends at least at much on their histological as on their molecular structure, even at so high a frequency as 5 Mc/s. It is found that ram's horn is transversely isotropic about the radial direction, and that rhinoceros horn is at least approximately transversely isotropic about the fibre direction, and this difference is correlated with the histological structure of the two materials. The approximate values of the elastic constants corresponding to the propagation of dilatational waves along the r, θ, and z directions respectively are: for ram's horn: 0·85, 1·01, and $1{\cdot}01 \times 10^{11}$ dyn cm^{-2}; for rhinoceros horn: 0·80, 0·87, and $1{\cdot}09 \times 10^{11}$ dyn cm^{-2}.

The anisotropy of baleen also appears to be correlated with its histological structure. The elastic constants determined for propagation parallel to θ and z respectively are approximately 0·80 and $1{\cdot}06 \times 10^{11}$ dyn cm^{-2}.

Some very approximate data are also presented for the cortex of African porcupine quill.

VIII. Acknowledgments

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HEAT TOLERANCES OF AUSTRALIAN MONOTREMES AND MARSUPIALS

By Kathleen W. Robinson*

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Summary

Heat tolerances of monotremes and marsupials have been compared by exposing the animals in a hot room, on different days for 7-hr periods, to various combinations of dry-bulb temperature ranging from 86° to 108·5°F with absolute humidity from 15 to 40 mm v.p.

Changes in their various physiological responses during heat exposure were examined.

Animals studied included the echidna, platypus, bandicoot, possum, cuscus, koala, wallaby, and wallaroo.

Results show an evolutionary trend in homeothermism. The monotremes possess the most primitive type of heat regulation, relying primarily on metabolic regulation to maintain body temperature. Sweat glands on the snout and over the body of the platypus give it a slight advantage over the echidna in hot, dry atmospheres.

Marsupials show a distinct advance on monotremes in heat regulation in employing both respiratory and non-respiratory evaporative mechanisms to an increasing degree, in the following ascending order: bandicoot, possum, cuscus, koala, wallaby, wallaroo. The cuscus, which inhabits the coastal regions of tropical Queensland and New Guinea, has a more effective evaporative mechanism than the more widely distributed possum.

Animals which rely to a large extent on evaporative methods for heat disposal are more distressed in humid atmospheres than those which favour other methods of heat disposal.

I. Introduction

Towards the close of the last century, isolated observations were made on the body temperatures of monotremes and marsupials (Miklouho-Maclay 1884; Semon 1894; Vernon 1898). Then appeared two important papers, first that of Sutherland (1896) recording body temperature at different air temperatures, and later Martin's (1903) valuable metabolic studies during heat exposure.

As an outcome of these experiments, several investigators, notably Sutherland (1896), Vernon (1898), and Martin (1903, 1930), pointed out that these two groups of animals present a stage of physiological development intermediate between the close approaches to homeothermism of the higher mammals and the rudimentary indications of this in lower vertebrates. Further details were added when daily body temperature variations in the echidna were recorded by Wardlaw (1918).

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Although in recent years some detailed studies of heat regulation have been carried out on American marsupials (Morrison 1946; Schmidt-Nielsen and Schmidt-Nielsen 1952), there has been a dearth of similar work in Australia. Since Australian lower mammals, particularly the monotremes, hold such an interesting position in the phylogenetic development of thermo-regulation, it was felt that this deficiency of knowledge should be remedied and to meet this need the present study was undertaken. In this investigation the heat tolerances of monotremes and marsupials are compared and changes in several physiological parameters during heat exposure are recorded.

II. METHODS

(*a*) *Atmospheres*

The animals were exposed for 7 hr, twice a week, to 11 combinations of dry-bulb temperatures varying from 86° to 108·5°F with absolute humidities between 15 and 40 mm v.p. Air movement remained constant at an average velocity of 60 ft/min.

(*b*) *General Conditions*

The animals remained in the air-conditioned room in which the required temperature and humidity had been produced for 7 hr or until a rectal temperature of 101°F for monotremes or 103°F for marsupials had been reached. Just above these temperatures signs of heat stroke begin to appear (Robinson and Lee 1946). Free access to water was allowed during the heat exposure period. The animals were fed before but not during the experimental period.

The species, as classified by Troughton (1941) and Wood Jones (1923), and number of animals used in the experiment were:

Monotremes:

Platypus (*Ornithorhynchus anatinus*): four animals
(two male, two female).
Echidna (*Tachyglossus aculeatus*): four animals
(two male, two female).

Marsupials:

Bandicoot (*Perameles nasuta*): five animals
(three male, two female).
*Possum (*Trichosurus caninus*): four animals
(two male, two female).
Cuscus (*Spilocuscus nudicaudatus*): five animals
(one male, four female).
Koala (*Phascolarctos cinereus*): two animals
(two female).
Wallaby (*Petrogale penicillata*): one animal
(female).

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* The initial 'o' has been omitted deliberately to distinguish it (a diprotodont) from the American or true opossums, which belong to the polyprotodont sub-order of marsupials (Troughton 1941).

Table 1

MEAN 9 A.M. READINGS WITH STANDARD DEVIATIONS

	Monotremes		Marsupials					
	Echidna	Platypus	Bandicoot	Possum	Cuscus	Koala	Wallaby	Wallaroo
No. animals	4	2	5	4	5	2	1	1
No. observations	39	10	49	46	50	22	11	5
Body wt. (kg)	3·8±0·3	0·75±0·12	4·6±1·5	3·5±0·9	3·2±1·1	3·4±0·2	16·4±0·8	37·2±0·5
Rectal temp. (°F)	83·1±3·3	86·0±2·7	95·7±1·2	94·5±1·5	94·8±1·5	97·0±1·2	97·8±1·4	93·4±0·4
Rectal temp. (°C)	28·4±1·8	30·0±1·5	35·4±0·6	34·7±0·8	34·9±0·8	36·1±0·6	36·5±0·8	34·1±0·2
Pulse rate/min	96±13	115±5	158±24	226±25	142±43	79±10	73±11	64±8
Resp. rate/min	11±3	19±1	82±25	75±24	92±26	38±9	64±8	48±10

Wallaroo (*Macropus robustus*): one animal
(female).

All animals were healthy young adults and their body weights are set out in Table 1. Food supplied to the platypus consisted of prawns and worms; and a pool with bank of sand was available for swimming and burrowing during non-experimental periods. The echidnas were fed bread and milk, finely minced meat, and hard-boiled eggs. A vegetable diet comprising gum leaves, fruit, and root and leafy vegetables was provided for the marsupials.

The following observations were made immediately before the animals entered the hot room and at hourly intervals thereafter: rectal temperature—by clinical thermometer; pulse rate—by stethoscope over the heart or manual palpation of the femoral artery (wallaby, wallaroo); respiratory rate—by observing movements of the flank. Animals were weighed hourly on a Sauter balance sensitive to 1 g. Urine and faeces were collected; the water consumed was measured; and evaporative weight loss was determined by difference.

III. Results

(*a*) *Rectal Temperature*

The mean 9 a.m. ante-room rectal temperature for each species, with standard deviation and number of estimations, is given in Table 1. The experiments were carried out in autumn, winter, and spring, when the 9 a.m. dry-bulb air temperatures ranged from 57° to 77°F.

The values fall into two distinct groups: the monotremes with a low body temperature and a considerable range of variation (S.D. ±2·7° to ±3·3°F), the marsupials with a higher body temperature and a restricted variation (S.D. ±0·4° to ±1·5°F).

As with most other animals when exposed to heat (Lee and Robinson 1941), the rectal temperature rose during the first 2 hr, the rise being much greater in monotremes (3·5°F/hr) than in marsupials (1°F/hr) (Fig. 1*A*, *B*). By comparison, Lee and Robinson found that in Eutheria, under resting conditions, rectal temperature rises only with shade air temperatures above 90°F, and then the rate of rise in the first 2 hr varies between 0·5° and 1°F/hr. Unless the conditions were too severe, a new equilibrium between heat gain and loss was reached by marsupials at a higher rectal temperature than normal. This equilibrium was approached but rarely achieved by the monotremes.

The effect on heat tolerance of varying dry-bulb temperature at constant humidity and varying humidity at constant dry-bulb temperature is shown in Table 2. It will be noted that heat tolerance is much lower in monotremes than marsupials, and in addition the separate effects of dry-bulb temperature and humidity differ in the two subclasses. The monotremes are greatly stressed by rising dry-bulb temperatures, whilst rising humidity at 90·5°F is fairly well tolerated. With the marsupials, both dry-bulb temperature and humidity play a part in producing heat stress but the evolutionarily more primitive types,

TABLE 2
HEAT TOLERANCES*

	Atmosphere		Monotremes		Marsupials					
	Dry-bulb Temperature (°F)	Humidity (mm v.p.)	Echidna	Platypus	Bandicoot	Possum	Cuscus	Koala	Wallaby	Wallaroo
Varying dry-bulb temperature at constant humidity	86	20		53						
	90·5	20		14						
	95	20		−57						
	86	30	23	53	96	63	83	85	88	81
	90·5	30	2	20	84	67	79	79	92	
	95	30	−128		73	62	64	73	84	
	99·5	30			60	53	60	69	83	
	104	30	−170		42	45	58	65	77	58
	108·5	30			5	37	52	52	76	
	93	40								59
	95	40	−120							56
	101	40								22
Varying humidity at constant dry-bulb temperature	90·5	15	13							
	90·5	17	11							
	90·5	20		14						
	90·5	25	13							
	90·5	30	2	20						
	95	30	−128							
	95	40	−102							
	104	15			59	56	—	74	83	
	104	20			58	46	59	71	87	
	104	25			44	34	54	71	73	
	104	30	−170		42	45	58	65	77	
	104	35			38	36	49	61	40	
	104	40			25	28	38	51	27	

* Heat tolerance coefficient = 100 − (average rectal temp. in room − average ante-room rectal temp.) × 10.

without efficient evaporating cooling, are more affected by dry-bulb temperature than are the more advanced types. The somewhat primitive bandicoot is more stressed by high dry-bulb temperature than humidity; the possum, cuscus, and koala equally affected by both; whilst humidity, particularly in the higher ranges, is more distressful to the wallaby, which is placed high on the evolutionary scale.

R values, which represent the areas under the reaction curves, have been used to compare the effect on each species of a particular combination of dry- and wet-bulb temperature. The values were derived from the following expression:

$$R = (a/2 + b + c \ldots\ldots + p/2)\, I,$$

when $a, b, c, \ldots\ldots p$ are successive average measurements at equal time intervals, I, commencing with ante-room value assumed at zero time.

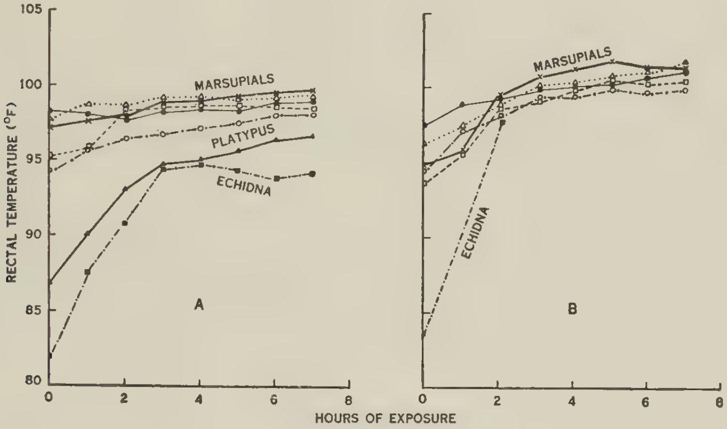

Fig. 1.—Effect of (*A*) 90·5°F dry-bulb temperature and 30 mm. v.p., and (*B*) 104°F dry-bulb temperature and 30 mm v.p. on rectal temperatures of monotremes and marsupials.

If the animal had to be removed before the period of exposure was complete, the adjusted *R* value was obtained by extrapolation as follows:

$$R = (a/2 + b + c \ldots\ldots + l/2)\, I + lt + t^2 \frac{(l-k)}{2I},$$

where k and l are the second last and last average measurements respectively, and t is the unexpired portion of the exposure.

R values for rectal temperatures of monotremes and marsupials are recorded in Tables 3 and 4.

Both echidna and platypus withstood exposure to 90·5°F for 7 hr but, when the dry-bulb temperature was increased to 95°F, the regulating mechanism became inadequate and body temperature rose rapidly to 101°F.

The bandicoot was the only marsupial unable to withstand 104°F 30 mm v.p. for 7 hr. With the exception of the Macropodidae, the remainder of the

Table 3

R VALUES FOR MONOTREMES OVER 7-HR EXPOSURE PERIOD

Figures in brackets have been weighted as described in the text. The number above the bracket indicates how many animals had to be removed with a body temperature of 101°F before 7 hr had elapsed; and that below, the hours that the animals remained in the room. When the animals had to be removed before sufficient figures required to estimate evaporative loss had been obtained, a blank appears in the column. An approximate mean value for rectal temperature, pulse rate, or respiratory rate may be obtained by dividing the appropriate figure by 7, and for evaporative loss by dividing by 6.

	Atmosphere		Rectal Temp. (°F)		Pulse Rate (no./min)		Respiration Rate (no./min)		Evaporative Weight Loss (g/kg)	
	Dry-bulb Temp. (°F)	Humidity (mm v.p.)	Echidna	Platypus	Echidna	Platypus	Echidna	Platypus	Echidna	Platypus
Varying dry-bulb temperature at constant humidity	86	20		634·8		801		135		35
	90·5	20		662·0		860		118		58
	95	20		$(712\cdot1)^{2}_{3\cdot5}$		(1026)		(185)		—
	86	30	635·3	634·8	664	809	141	130	6	40
	90·5	30	650·6	657·7	800	759	257	137	5	58
	95	30	$(741\cdot6)^{4}_{2\cdot8}$		(1088)		(472)		—	
	104	30	$(770\cdot9)^{4}_{2\cdot2}$		(1182)		(992)		—	
Varying humidity at constant dry-bulb temperature	90·5	15	642·4		738		151		7	
	90·5	17	644·1		798		169		13	
	90·5	20		662·0		860		118		58
	90·5	25	642·4		764		143		10	
	90·5	30	650·6	657·7	800	759	257	137	—	58
	95	30	$(741\cdot6)^{4}_{2\cdot8}$		(1088)		(472)		—	
	95	40	$(723\cdot0)^{4}_{2\cdot9}$		(1014)		(449)		—	

group had to be removed with signs of heat stroke at a dry-bulb temperature of 108·5°F.

It is interesting to note the superiority of the cuscus over the possum in hot atmospheres (Table 4 and Fig. 1). Both animals belong to the Phalangeridae.

(*b*) *Pulse Rate*

Normal values for pulse rates at rest in the absence of heat stress are given in Table 1. As with most poorly sweating animals (Lee and Robinson 1941), the pulse rate of monotremes and marsupials shows little rise with continued exposure to a room temperature which produces a marked rise in rectal temperature. Neither change in humidity nor change in dry-bulb temperature has a significant effect (Tables 3 and 4).

(*c*) *Respiratory Rate*

Monotremes respire at a much slower resting rate than marsupials (Table 1). Martin (1903) showed that respiratory rate changed little when the monotremes were exposed for short periods (30 min) to different temperatures. Even after 7 hr exposure in our experiments, the rate of respiration remained slow, except at the highest range of temperature. Open-mouthed panting did not occur.

As a marked contrast, the rate of breathing increases rapidly in marsupials during heat exposure, particularly in hot, dry atmospheres (Table 5). With more stressful conditions, the mouth opens slightly to allow increased evaporation from the buccal cavity. Panting is minimal in the cuscus but particularly marked in the wallaroo, where it resembles the panting of a dog.

(*d*) *Evaporative Weight Loss*

Weight loss has been estimated as g/kg body weight in order that a comparison may be made between species.

The following findings are recorded in Tables 3 and 5:

(i) The platypus has a higher rate of evaporation than the other mammals.
(ii) Monotremes show very little change of weight with rising temperature.
(iii) In marsupials, evaporative loss increases markedly as dry-bulb temperature rises but remains fairly constant with changing humidity. This correlates highly with respiratory rate. The cuscus, however, does not lose water proportionally to respiratory rate.

The apparent increase in values with some of the highest humidities may be explained by increased loss through drooling.

(*e*) *Behaviour*

Details of the behaviour of echidna, bandicoot, possum, cuscus, and wallaroo in hot atmospheres are described in an earlier publication (Robinson and Lee 1946).

Table 4

R VALUES FOR RECTAL TEMPERATURE (°F) AND FOR PULSE RATE FOR MARSUPIALS OVER 7-HR EXPOSURE PERIOD

Figures in brackets have been weighted and the numbers outside the brackets are as described in Table 3. An approximate mean value for rectal temperature or pulse rate may be obtained by dividing the appropriate figure by 7

	Atmosphere		Bandicoot		Possum		Cuscus		Koala		Wallaby		Wallaroo	
	Dry-bulb Temp. (°F)	Humidity (mm. v.p.)	Rect. Temp.	Pulse Rate	Rect. Temp.	Pulse Rate	Rect. Temp.	Pulse Rate	Rect. Temp.	Pulse Rate	Rect. Temp.	Pulse Rate	Rect. Temp.	Pulse Rate
Varying dry-bulb temperature at constant humidity	86	30	672·6	805	687·4	1489	675·8	1093	689·7	667	693·1	588	693·1	356
	90·5	30	681·3	880	684·4	1559	678·5	972	693·7	778	689·8	484		
	95	30	688·6	1066	688·3	1523	688·6	989	697·7	762	695·5	560		
	99·5	30	697·6	955	694·6	1467	691·3	758	700·4	786	696·1	482		
	104	30	$(710{\cdot}7)^{3}_{6{\cdot}0}$	(1005)	700·3	1535	693·3	1061	703·4	759	700·5	512	683·4	404
	108·5	30	$(736{\cdot}6)^{3}_{4{\cdot}0}$	(1279)	$(705{\cdot}5)^{2}_{5{\cdot}3}$	(1578)	$(679{\cdot}0)^{1}_{6{\cdot}3}$	(1178)	$(712{\cdot}5)^{2}_{3{\cdot}0}$	(754)	701·1			
	93	40											682·6	440
	95	40											684·6	444
	101	40											708·2	656
Varying humidity at constant dry-bulb temperature	104	15	698·4	980	692·3	1470			697·4	632	696·5	504		
	104	20	699·3	994	$(699{\cdot}3)^{1}_{6{\cdot}0}$	(1574)	692·2	931	699·4	739	693·7	483		
	104	25	$(709{\cdot}4)^{1}_{6{\cdot}4}$	938	$(707{\cdot}6)^{2}_{4{\cdot}8}$	(1561)	695·7	1092	699·0	671	703·5	518		
	104	30	$(710{\cdot}7)^{3}_{6{\cdot}0}$	(1005)	$(700{\cdot}3)^{2}_{5{\cdot}8}$	(1535)	693·3	1061	703·4	759	700·5	512		
	104	35	$(713{\cdot}6)^{3}_{5{\cdot}0}$	(1080)	$(706{\cdot}1)^{2}_{5{\cdot}8}$	(1585)	699·4	1071	706·3	782	$(726{\cdot}5)_{2{\cdot}4}$	(638)		
	104	40	$(722{\cdot}8)^{5}_{3{\cdot}9}$	(1156)	$(711{\cdot}6)^{2}_{5{\cdot}1}$	(1491)	$(706{\cdot}9)^{2}_{5{\cdot}8}$	(1190)	$(713{\cdot}5)^{2}_{3{\cdot}0}$	(792)	$(735{\cdot}9)_{1{\cdot}75}$	(616)		

The outstanding feature of the behaviour of the platypus when exposed to heat is drowsiness. It lies at first curled up in a ball, but as body temperature rises to 95°F its posture changes. It then rolls over on to its back with under surface exposed and legs outstretched but still remains inactive. There is a slight increase in respiratory rate but no attempt at panting. With higher body temperatures, the platypus becomes very restless and a slight tremor develops over its entire body, suggesting some impairment of neuro-muscular coordination. During heat exposure, this animal neither salivates nor increases evaporation from the body surface by coat licking.

The koala curls up and remains asleep at external temperatures of 86°F. At higher temperatures, which cause only a slight rise in body temperature, respiratory rate increases rapidly. When body temperature approaches 99°F, it begins licking its front and hind paws, the pads particularly, and it rubs their wet surfaces over its face. At this temperature, it also sits back like the possum, exposing its chest and abdomen. Later panting commences but the mouth opens only slightly and, although there is some dribbling on to the chin and abdomen, it is less profuse than that of the possum. Troughton claimed that the koala never drinks water but in these experiments, when body temperature rose to 101°F, it would repeatedly move over to its drinking vessel and lap small quantities. Evidently this is a procedure adopted only under severe heat stress.

When first exposed to a hot climate, a wallaby sits quietly though less drowsily than the other marsupials. Increased respirations occur after slight rise in body temperature and, when this reaches 100°F, rapid respirations are replaced by open-mouthed panting. Around this temperature, the animal continually licks its forearms and body and wipes its face with the wet paws. At 101°F body temperature, dribbling is profuse. The wallaby repeatedly drinks small quantities of water, a 16-kg wallaby consuming 150-250 ml in the 7-hr exposure period. At a body temperature of 103°F heat staggers begin to develop in its hind legs. The behaviour of wallaroos closely resembles that of wallabies in a hot environment.

IV. DISCUSSION

These results are in agreement with Martin's findings that monotremes exhibit some degree of homeothermism although the stability of their heat regulating mechanism can be easily upset at air temperatures above 90·5°F. The platypus is somewhat better regulated than the echidna in this respect.

Monotremes do not pant and are devoid of vasomotor adjustments, as shown by the circulatory experiments of Martin (1903) and pulse rate figures in this study. The only method available therefore to prevent over-heating is metabolic regulation—the reduction of heat production (Martin 1903). This would thus seem to be the most primitive type of heat-regulating mechanism used by mammals. It necessitates an inactive existence during the daytime. The animals spend this period in burrows where air temperatures are lower (rarely exceeding 86°F, as shown by Vorhies (1945)), and so are able to

Table 5

R VALUES FOR RESPIRATORY RATE/MIN AND FOR EVAPORATIVE WEIGHT LOSS (gk/g) FOR MARSUPIALS OVER 7-HR EXPOSURE PERIOD

Figures in brackets have been weighted as described in the text. Blanks are explained in Table 3. Evaporative loss was not measured in the koala. An approximate mean value for respiratory rate or evaporative loss may be obtained by dividing the appropriate figure by 7 or 6 respectively

	Atmosphere		Bandicoot		Possum		Cuscus		Koala	Wallaby		Wallaroo	
	Dry-bulb Temp. (°F)	Humidity (mm v.p.)	Resp. Rate	Evap. Loss	Resp. Rate	Evap. Loss	Resp. Rate	Evap. Loss	Resp. Rate	Resp. Rate	Evap. Loss	Resp. Rate	Evap. Loss
Varying dry-bulb temperature at constant humidity	86	30	329	2·8	490	12·6	521	6·9	254	685	17·8	392	19·1
	90·5	30	384	5·0	585	11·4	326	10·6	940	686	19·4		
	95	30	758	12·4	564	16·3	361	12·8	1140	1039	16·1		
	99·5	30	1113	12·6	852	20·0	707	15·0	1343	1482	25·2		
	104	30	(1364)	(21·5)	1121	37·1	730	18·1	1436	1607	36·2		
	108·5	30	(1926)	—	(1348)	(39·4)	(935)	(40·0)	(1570)	1781	39·6	1892	30·9
	93·0	40										1828	8·7
	95	40										1956	20·4
	101	40										1940	15·6
Varying humidity at constant dry-bulb temperature	104	15	927	14·1	754	31·1			1157	1178	46·6		
	104	20	1168	13·9	(1231)	(36·0)	732	21·3	1320	1414	34·9		
	104	25	(1561)	(18·0)	(1569)	(33·4)	626	17·8	1334	1464	36·5		
	104	30	(1364)	(21·5)	(1121)	(37·1)	730	18·1	1436	1607	36·2		
	104	35	(1531)	(32·4)	(1458)	(31·4)	1012	18·8	1543	(2027)	—		
	104	40	(1949)	—	(1887)	(36·8)	(1038)	(22·5)	(1623)	(1850)	—		

survive the hot Australian summers. Since evaporation is not an important cooling mechanism in monotremes, hot, wet climates with a lower dry-bulb temperature are preferred.

The slight superiority of the platypus over the echidna in heat regulation may be explained by differences in evaporative loss. The echidna has no sweat glands, whilst these are generously scattered over the snout of the platypus and in addition one apocrine gland opens into the follicle of each of the large hairs (Wood Jones 1923; Hardy 1944). Since neither animal alters respiratory rate significantly during heat exposure, the increased water evaporated from the platypus in hot, dry atmospheres is to be interpreted as increased activity of these sweat glands. Since they are confined to such a small area, their effectiveness is very limited.

Marsupials show a distinct advance in homeothermism, being able not only to vary heat production (Martin 1903) but heat loss as well. The higher body temperature would to some extent aid heat transfer by radiation but by far the greater loss arises from increased powers of evaporation (*a*) from the respiratory tract, and (*b*) from the body surface. The additional evaporation which takes place from the buccal mucosa during open-mouthed panting is evident when measurements of respiratory rate and evaporative weight loss are compared. Although, histologically, sweat glands can be demonstrated over the entire body surface of marsupials (Bolliger and Hardy 1945; Hardy 1947), they are of a primitive apocrine type and their poor functioning power is shown by the readiness with which these animals use saliva to aid evaporation, particularly from the abdomen, limbs, and tail. The cuscus appears to use a non-respiratory type of evaporative mechanism extremely well. Comparing it with the possum (a member of the same family), we find that, in hot, dry climates, evaporative weight loss rises much more steeply than does increase in respiratory activity. This is associated with a better heat tolerance response. It is interesting to note that the cuscus is restricted to the hot coastal regions of tropical Queensland and New Guinea whilst the possum abounds in more temperate parts. It is likely that this difference in evaporative power has been developed over the years as an adaptation to the thermal stress of the tropics. It could arise in one or both of two ways:

(*a*) By a more efficient sweating mechanism; or

(*b*) By a superior method of salivary application.

Histological studies should yield some information on the sweat glands, but they have not yet been examined in sufficient number. Skin sections taken from a single specimen of cuscus showed a fair number of sweat glands (one associated with each primary hair follicle) and these seemed to be functioning actively (Hardy, personal communication 1953). That sweating is probably the true explanation is suggested from evidence of Hardy (1947) that bandicoots from tropical districts tend to show better-developed sweat glands than those from colder areas. The constant licking of the bare tail would favour salivary cooling techniques whilst the peculiar posture adopted and the large water intake when body temperature rises would support both concepts.

The degree of development in marsupials of an evaporative mechanism for heat removal parallels structural evolutionary trends. Heat tolerance correlates with this evolutionary picture, increasing in the following ascending order: bandicoot, possum, cuscus, koala, wallaby, wallaroo. Evaporative cooling is more effective in hot, dry than in hot, wet climates and animals which rely most upon it are most affected by rising humidity. This method of heat transfer finds its highest development in man and the horse.

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GROWTH OF THE MOUSE COAT

III. PATTERNS OF HAIR GROWTH

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Summary

Hair growth cycles were observed for a period of 60 days in 10 Naked mice of different ages. The duration of the total hair cycle varied according to position and increased from head to tail. Within each position over all ages the duration was constant. These facts explain the pattern of growth cycles in Naked mice.

I. INTRODUCTION

The cyclical replacement of the mouse coat is a well-established fact, and has been studied in detail by Dry (1926), Dannel and Kahls (1947), Fraser (1951), Fraser and Nay (1953), and several others. Dry examined microscope preparations of hair samples from different sites and deduced the position of bands of hair growth from the stages of development of these samples. He achieved very exact information but his analysis suffered from (*a*) the heterogeneity of the mice which he used, (*b*) the necessity of killing each animal to obtain the hair samples, and (*c*) the short range of the analysis, which extended only to the fourth hair cycle. Dannel and Kahls (1947) made drawings of the internal surface and so could also make only one observation per animal. Fraser (1947) measured the rate of growth in length of the different types of hairs which occur in the first coat. He obtained accurate estimates of the ages of initiation and cessation of growth of the first coat in four genetically defined stocks of mice. However, his observations were again limited to one per individual, and to the first hair cycle. He suggested that these difficulties could be avoided by utilizing the Naked gene (Lebedevsky and Dauvert 1927; see also Grüneberg 1952) which causes ordinary coat hairs to break and be shed just before the completion of their growth. Such mice differ from normal mice in exhibiting a covering of coat hairs only on those regions which are actively in the growth phase. The advantages of using Naked mice are that observation of the position of hair bands is very simple and fast (Fraser and Nay 1953), and that repeated observations can be made on the same individual.

II. METHODS AND MATERIAL

Dannel and Kahls (1947) traced the positions of hair bands on a silhouette representing the whole outstretched skin of an animal. They did not transform

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their observations onto a numerical scale. Fraser and Nay (1953) also used standard silhouettes to record positions of hair bands in Naked mice. They used a top and a side view, and it was found by Turner (appendix to Fraser and Nay 1953) that positions of hair bands could be estimated equally from the top or side silhouettes. The top view has therefore been discarded.

The position of a hair band is determined along a line running from head to tail, along the side, which is divided into 64 segments, and position is measured from the head (0) to the root of the tail (64).

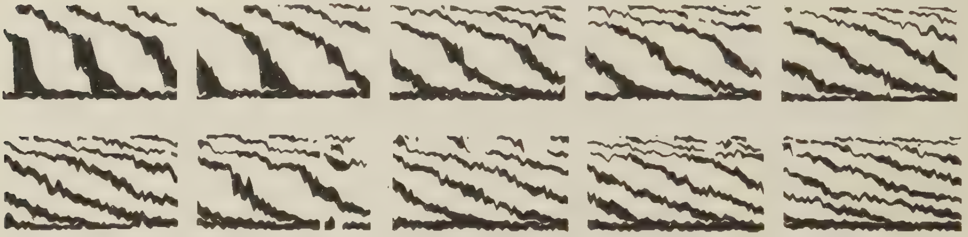


Fig. 1.—Hair growth cycles plotted against age and position separately for each of the 10 mice. Each graph extends over 60 days, from the ages given in the text.

The stock of mice used was the NA stock, which is a highly inbred subline of the Strong A strain kept segregating for the *Na* gene (Fraser and Nay 1953). Ten adult male mice, selected at random, were drawn daily for a period of 60 days. The ages and weights of these mice at the beginning of observations were:

(1)	73 days	20·7 g
(2)	78 days	23·6 g
(3)	123 days	26·5 g
(4)	140 days	22·5 g
(5)	150 days	23·1 g
(6)	181 days	24·5 g
(7)	195 days	20·9 g
(8)	217 days	24·6 g
(9)	227 days	25·4 g
(10)	340 days	24·9 g

The drawings (600) were scored for positions of the hair bands, and these were then plotted against age, to allow study of the changes of position (Fig. 1). Two measurements were extracted from these graphs.

(i) *Duration of Total Cycle* (A-B).—This is the interval between the successive initiations of hair growth phases in a specified region. On our diagrams this is the interval between the passage of two successive anterior (advancing) edges of hair bands over a specified region. The measurement (*A-B*) includes both the growth and the rest phases.

(ii) *Duration of the Growth Phase* (C-D).—The interval between initiation and cessation of hair growth, on a specified region. Both *A-B* and *C-D* were measured to ± ½ day. They are illustrated in Figure 2.

Since observations were made of different mice at the same age, their graphs overlap; they were therefore superimposed, and the advancing (anterior) edge of hair cycles traced onto a single diagram (Fig. 3). A mean value of the positions of the anterior edge of successive hair cycles was determined and plotted in Figure 4.

III. Results

(*a*) A-B. *Duration of Total Cycle*

A-B was measured for several positions and the age at each measurement taken as the position of *A* (Fig. 2). No change of *A-B* with age was found over the range of ages included in our data. This is illustrated in Figure 5. The duration of a total cycle does vary with position, increasing from head to tail. This is shown in Figure 6.

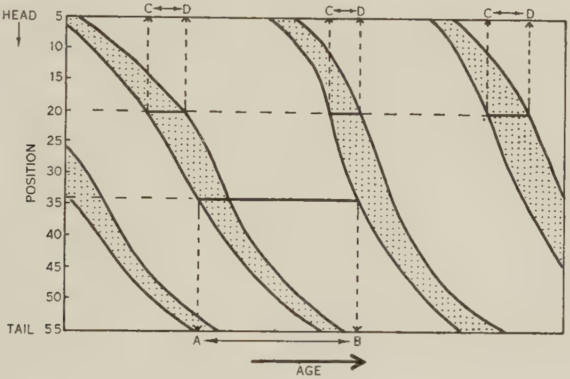


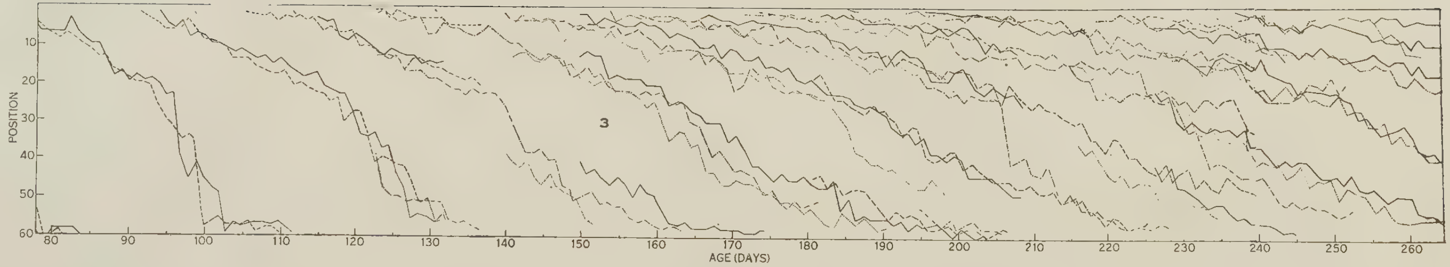
Fig. 2.—A schematic pattern of hair growth cycles to show how *A-B* and *C-D* are obtained from the data in Figure 7.

(*b*) C-D. *Duration of Growth Phase*

Measurements of *C-D* were made in the same way as for *A-B*. Again no change of *C-D* with age and a marked increase from head to tail were found (Figs. 7 and 8).

The average values of *A-B* and *C-D* for different positions are given in Figure 9. A feature of this diagram is the demonstration that the duration of the resting phase also increases from head to tail.

The diagram (Fig. 3) in which data from the different mice were superimposed shows a surprising uniformity of the total pattern. This can be explained by the genetical uniformity of the stock and by the environmental variation of the pattern being low. Only one mouse (7) is out of step, showing a more juvenile pattern than would be expected. This mouse became sick and emaciated towards the end of the observation period, and died shortly afterwards. Results obtained from this mouse were not included in the average diagram (Fig. 4). The diagram in which the positions of hair bands were averaged (Fig. 4) illustrates several features of the total pattern which require



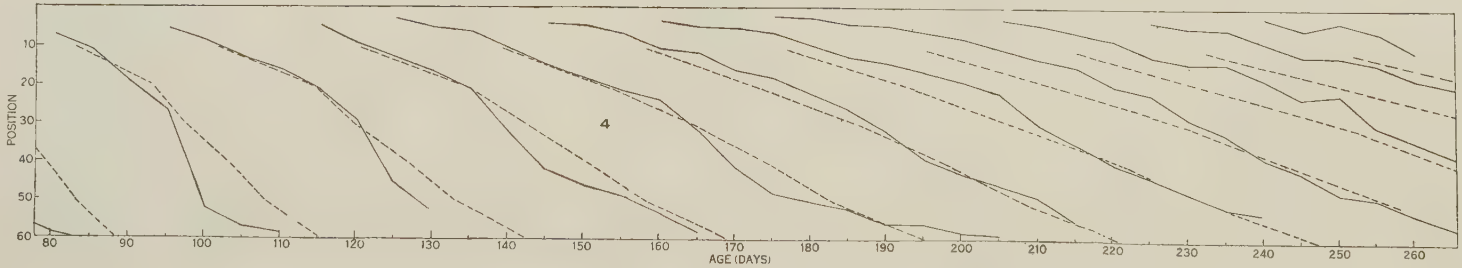


Fig. 3.—Plots of the position of the advancing (anterior) edge of the hair bands, overlapped for each of the 10 mice.

Fig. 4.—Plot of the position of the advancing (anterior) edge of hair bands, averaged over all mice, and superimposed on the theoretical pattern (broken line).

explanation. The slope of the movement of hair bands decreases markedly with age. The width of a hair band decreases markedly with age. The number of hair bands occurring simultaneously on a single animal increases with age. This is very well marked in the comparisons of animals 1 and 8 (Fig. 1).

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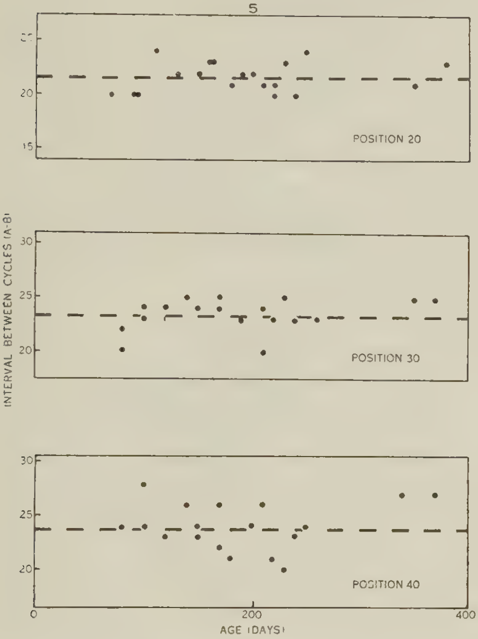

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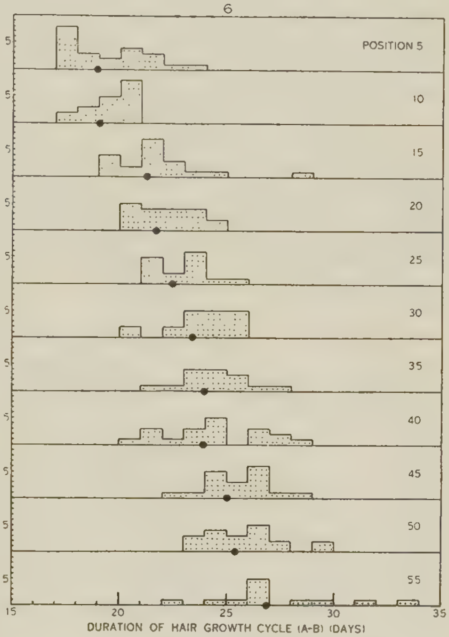

Fig. 5.—Duration of total cycle (*A-B*) plotted against age for positions 20, 30, and 40.

Fig. 6.—Frequency histograms of *A-B* for different positions, taken over all mice and ages.

IV. Discussion

It has been shown that although the duration of the resting and growth phases increases from head to tail, yet they are invariant with age. These two facts supply the explanation of all the features of the pattern of hair cycles, and this is clearly demonstrated by calculating a theoretical pattern of hair cycles derived solely from our estimates of the values of *A-B* which were given in Figure 6. The construction of such a theoretical pattern has been made for the anterior (advancing) edge in order that comparison can be made with the average values of the pattern, which are given in Figure 4.

Growth of the first coat begins about 8 days after birth (Grüneberg 1952) and although initiation on the head is earlier than initiation on the tail, the interval is only 1-2 days. We therefore assume that growth of the first coat commences simultaneously all over the body at 8 days after birth. The mean value of *A-B* at a given point gives the time of initiation of the second hair cycle at that position. By adding *A-B* for each position successively, the time of initiation of the third, fourth, fifth, and later periods of growth at each

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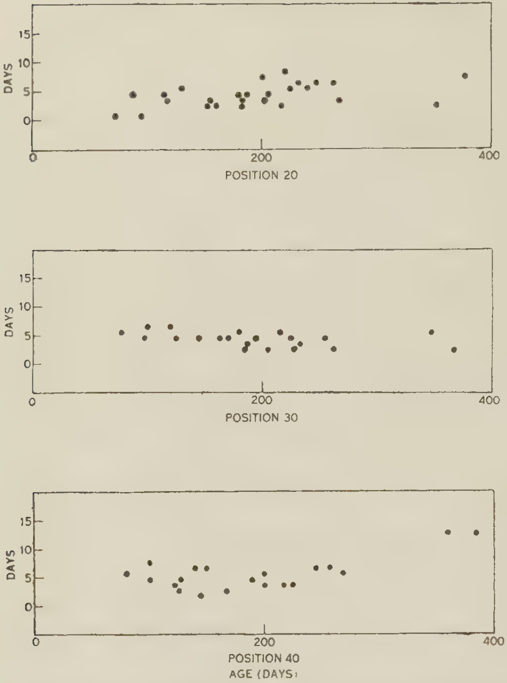

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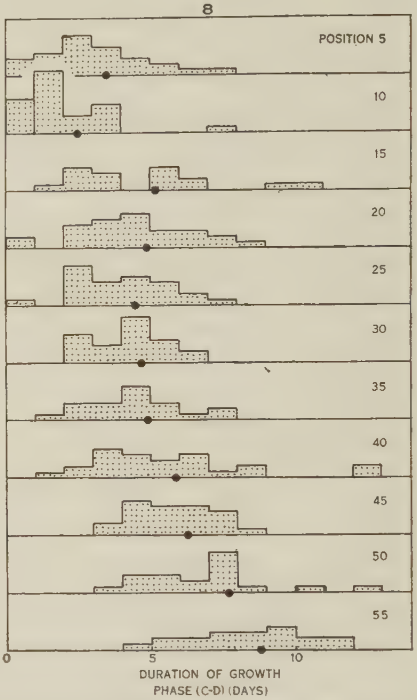

Fig. 7.—Duration of growth phase *C-D*, plotted against age for positions 20, 30, and 40 over all mice.

Fig. 8.—Frequency histograms of duration of growth phase *C-D*, for a series of positions, over all mice and ages.

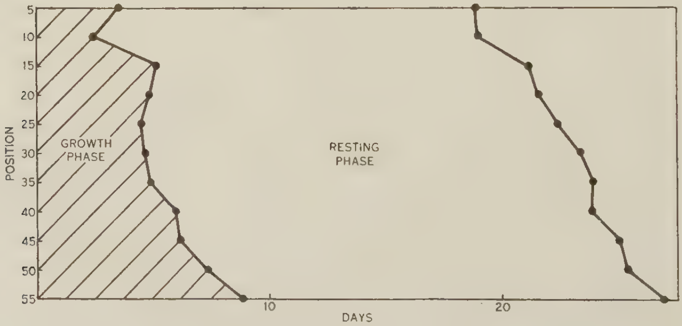

Fig. 9.—Average values of *A-B* and *C-D* for different positions to show the increase with position, from head to tail.

position can be found, and so a theoretical pattern of the hair cycles can be built up. This is shown by the broken lines in Figure 4. The agreement between actual and observed values is reasonable, and shows the validity of our assumption that the various features of the pattern can be explained (*a*) by the constancy of duration of hair cycles at each position, and (*b*) by the increase in duration of hair cycles in a gradient from head to tail.

There must be an early, probably pre-natal, determination of the constants of hair cycles. Each region follows its own pattern of growth and rest in slightly more time than the adjoining region cranial to it, and slightly less time than the adjoining region caudal to it. The mechanism can be analogized to a row of alarm clocks in which each clock runs at a slightly slower rate than the preceding one. After the first cycle the alarms will go off in an almost continuous sequence. However, as more and more cycles are run through, the alarms will go off in greater and greater disagreement.

It must be emphasized that although in adult males and unmated females the basis of the pattern of hair growth cycles is invariant with age, yet in pregnant females this pattern is consistently and characteristically disrupted (Lebedevsky and Dauvert 1927; Dannel and Kahls 1947; Fraser and Nay 1953).

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THE USE OF REGRESSIONS TO INCREASE HERITABILITY

By J. M. Rendel*

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Summary

The difficulties of applying correction factors to eliminate non-genetic variance in selective breeding work are discussed. The use of a second character as an index of environmental change is explored and related to the general formula for selection indices. Tibia length and body weight in mice are used to demonstrate points made in the theoretical discussions of the first part of the paper.

I. Introduction

When selection for a trait is practised in order to increase its expression in a population the expected rate of genetic change, ΔG, is proportional to the selection differential $\bar{\imath}$, the heritability of the trait in the population h^2, and the genetic variance $\sigma_G{}^2$, such that

$$\Delta G = \bar{\imath} h \sigma_G. \quad .. \quad .. \quad .. \quad .. \quad (1)$$

Whenever the genetic situation cannot be analysed into the effects of individual gene loci the breeder attempts to maximize expression (1) by manipulating those factors which he can control. $\bar{\imath}$, the difference in standard deviations between the mean of the population and the mean of those individuals which become parents, is determined by the fraction of the population saved as parents, and so by the rate of reproduction of the species; $\sigma_G{}^2$ is not readily changed except by crossing, a procedure which may undo the effects of selection; so most breeding programmes aim to maximize h^2 without reducing the other terms or increasing the generation length. Family selection and progeny testing can both be regarded as increasing h^2 by obtaining a measure of the parents selected that is a more accurate estimate of their genotype than a simple phenotypic score. The commonest method, however, is to reduce the phenotypic variation to a minimum by calculating environmental trends and correcting individual scores for the calculated effects of definable environmental effects. As $h^2 = \sigma_G{}^2/(\sigma_G{}^2 + \sigma_E{}^2)$ in its simplest form, anything which reduces $\sigma_E{}^2$ increases h^2.

II. Correction Factors

Before a correction can be applied it is first necessary to classify the individual as having been subjected to some environment with known phenotypic effect. It is simple to score an individual as belonging to a litter of one, two, three, or more litter mates, or as being in her first, second, third, or later lacta-

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tion, or as having had an interval of so many days between the first and second lactation. It is not always so easy to say that an individual has suffered from some infection, and difficult, if not impossible, to subdivide the infected individuals into classes of those which were more and those which were less severely infected. The number of possible trends which would have to be calculated to give a complete or nearly complete description of environmental effects is obviously large and would require much laborious computation to work out. But even when the computations have been made and where the environmental classes appear to be unequivocal there are difficulties in applying corrections which may not at first appear.

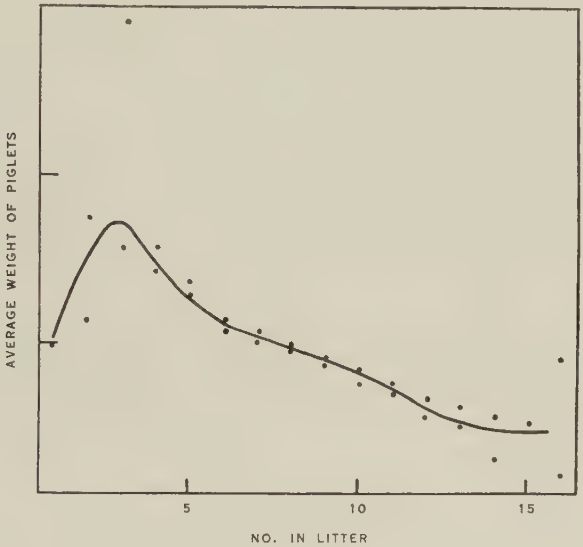

Fig. 1.—Effect of litter size on weight of pig at 3 wk (after Korkmann).

Let us take as an example the effect of number in the litter on 3-wk weight of piglets. It can be seen from Korkmann's (1947) data, Figure 1, that in general the larger the number in the litter the smaller the piglet. But as the number becomes very small a new trend makes its appearance and as the number decreases the weight of piglets also decreases. It can be shown for mice, where the trend of decrease in size with increase in number is slight (Table 1; Fig. 2), that as the number in the litter decreases, the variance increases by an amount which is greater than would be expected to result from the smaller sample size. It appears as though a new classification is required at this end of the curve. The old trend which affects litters of larger number continues to operate in so far as the biggest mice come from the smallest litters, but there is another sort of small litter in which the progeny are themselves much smaller than average. At this point a single correction on the basis of litter number is no longer applicable to all litters of a given number. Some litters are affected one way, and some another. It appears as though there were at least two kinds of litter, those which are small in number but otherwise normal, and those which are small in number because of some factor which affects the growth of mice adversely. If the same correction is applied to both sorts of litter on the grounds that they both belong to the class of litter which contains so many mice, some will be corrected insufficiently for a trend due to an environment to which they were subjected, and others will be corrected for a trend due to an environment to which they were not subjected, in a direction opposite to the one by which they were in fact affected.

Table 1

VARIANCE OF MEAN LITTER WEIGHT IN MICE CLASSIFIED ACCORDING TO NUMBERS IN LITTER

Number in the Litter	2	3	4	5	6	7	8	9	10
Mean wt. (g)	12·4 (12·4)	13·8 (12·1)	14·3 (13·1)	12·8 (11·4)	14·1 (11·6)	12·9 (11·4)	12·7 (9·8)	12·3	13·9
Variance of mean	10·15 (11·54)	6·41 (7·84)	4·48 (6·12)	2·08 (3·10)	1·24 (4·31)	3·42 (3·73)	1·41 (2·45)	2·25	0·85
Number of litters	4 (6)	6 (16)	7 (11)	10 (22)	8 (15)	9 (9)	7 (4)	4	2

Variance of weights of individuals of same litter = 1·68.

Figures in brackets come from crosses between pure lines and outbred mice, the remaining figures are from mice in this experiment.

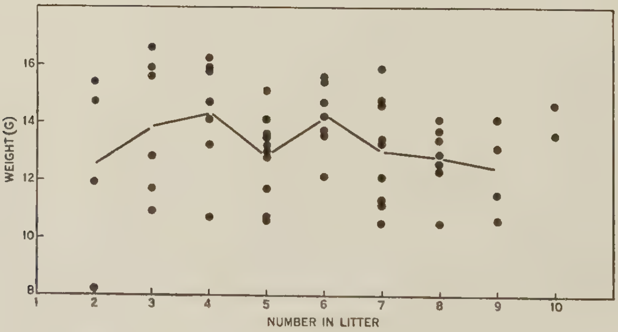

Fig. 2.—Effect of number in litter on weight of mice at 4 wk.

An index is required which will not only register the degree to which each individual has been exposed to a particular environment, but also integrate the effect of all possible environments into one figure. Hazel (1943) has suggested that the measure of one character can be used not only as an index of the genotype of the individual with respect to another character, but also of the environmental effects to which it has been exposed. Suppose the correlation between two characters is entirely environmental, then deviation from the mean population score in one is a measure of the extent to which the second has been affected by the environment and the regression of one character on the other can be used to correct for all those environmentally produced deviations which give rise to the correlation between them. The extent to which environmental variance in one character is removed by its regression on a second will depend on the closeness of the correlation between them.

III. Relationship Between Selection Index and Correction of One Character for Variation in Another

If two characters are measured on the same animal or plant the breeder must decide how best to use the information at his disposal when selecting parents for the next generation. In making his choice the weight he puts on the two scores will depend on the heritability of the characters and the economic value of an advance in them. The solution of the problem of how to weight the two traits to the best advantage was first put forward by Fairfield Smith (1937), using Fisher's discriminant functions, and later arrived at by Hazel (1943), using Sewall Wright's path coefficients. The problem and its solution are set out again here for convenience.

Suppose selection is being practised for two characters 1 and 2. The genotype of an individual may be written $G_1 + G_2$ and its phenotype $X_1 + X_2$. The economic value of an individual (H) depends on the money value a_1, a_2 of each character:

$$H = a_1G_1 + a_2G_2.$$

The best index on which to select, I, depends on the heritability of each character and its economic value, which must be taken into account when weighting X_1 and X_2:

$$I = S_1X_1 + S_2X_2.$$

The best index will be one for which H and I are maximally correlated. The problem is to find the weighting factors S_1 and S_2. The correlation of I with H can be written

$$R_{HI} = \frac{\text{cov } HI}{\sqrt{\text{var } H \times \text{var } I}} \quad \cdots \quad \cdots \quad \cdots \quad \cdots \quad (2)$$

The covariances and variances of H and I are simply expressed in terms of a, S, G, and X. The assumption is made that there is no correlation between environment and genotype so that, for example, the expression in the covariance term $S_1a_1X_1G_1$ is equivalent to $S_1a_1 \text{ var } G_1$ and the term $a_1S_2G_1X_2$ is equivalent to $a_1S_2 \text{ cov } G_1G_2$. If equation (2) is written out in full and converted to logarithms R_{IH} can be differentiated with respect to S_1 and S_2 and by equating these derivatives to zero, values for S_1 and S_2 found which maximize the correlation R_{IH}. As S_1 and S_2 are weighting factors, S_1 can be taken as unity and S_2 expressed in terms of S_1. S_2 then becomes

$$\frac{\text{cov } X_1X_2(a_1 \text{ var } G_1+a_2 \text{ cov } G_1G_2) - \text{var } X_1(a_1 \text{ cov } G_1G_2+a_2 \text{ var } G_2)}{\text{cov } X_1X_2(a_1 \text{ cov } G_1G_2+a_2 \text{ var } G_2) - \text{var } X_2 \, (a_1 \text{ var } G_1+a_2 \text{ cov } G_1G_2)}.$$

When all that is required of the second character, 2, is that it should indicate the environment to which 1 was exposed, it can be chosen so as to have no economic value itself, so that a_2 becomes zero and all terms containing a_2 disappear. If there is no genetic correlation between 1 and 2 all terms containing $\text{cov } G_1G_2$ also disappear, leaving $-\text{cov } X_1X_2/\text{var } X_2$. Thus when the second character is of no economic importance and when there is no genetic correlation between 1 and 2, or if it is desired not to increase 2, the regression of 1 on 2 is the best correction to use to make the most of information available.

$$I = X_1 - b_{1\cdot 2}X_2,$$

where $b_{1\cdot 2}$ is the regression of X_1 on X_2.

As it is usually difficult to get an accurate estimate of genetic correlation it is of interest to note what happens if the regression is used as a correction and there is some genetic as well as environmental correlation between the two characters. Reframing (**1**), we have before correction

$$\Delta G = \bar{i}h^2\sigma_p.$$

Correction removes a portion of the phenotypic variance due to regression equal to $r_{p_1p_2}{}^2\sigma_{p_1}{}^2$, where $r_{p_1p_2}$ is the correlation between the two phenotypes. Correction also removes $r_{G_1G_2}{}^2\sigma_{G_1}{}^2$ of the genetic variance of the first trait where $r_{G_1G_2}$ is the genetic correlation between them. After correction we find

$$\Delta G \text{ corrected} = \frac{\bar{i}\sigma_G{}^2(1-r_{G1G2}{}^2)\sigma_p(\sqrt{1-r_{p1p2}{}^2})}{\sigma_p{}^2(1-r_{p1p2}{}^2)}$$

$$= \frac{\Delta G(1-r_{G1G2}{}^2)}{\sqrt{1-r_{p1p2}{}^2}}$$

This is greater than ΔG so long as the genetic correlation is not large compared to the phenotypic one. Values of $r_{p_1p_2}$ below which correction decreases ΔG as shown in Figure 5 for different values of $r_{G_1G_2}$. It will be seen that in the absence of genetic correlation between two traits, and if one of them has no economic value, the selection index commonly used in plant and animal breeding leads to the conclusion that the best use of the second trait is as an indicator of the environment to which the first was subjected. That is, selection is made with the regression line as the base line and in this way h^2 is increased since $\sigma_E{}^2$ is reduced with respect to the character of economic value.

IV. EXPERIMENTAL MATERIAL

The measurements on which the method of correction discussed here has been tried out, came from a selection experiment using mice in which 15 males were mated each to four half sibs and the litters used to discriminate between the sires. The young mice were weighed at 4 wk, they were then killed and the tibia of one leg prepared after cooking in a pressure cooker for 5 min. The bone was projected onto a screen and the shadow measured. The length of the shadow, given in centimetres, is used in this analysis. Magnification was about 11¼ times. A total of 327 mice from 57 litters were finally measured. Mean weight was 13·25 g, and tibia length (shadow) 15·21 cm, so that actual tibias were about 1·35 cm long.

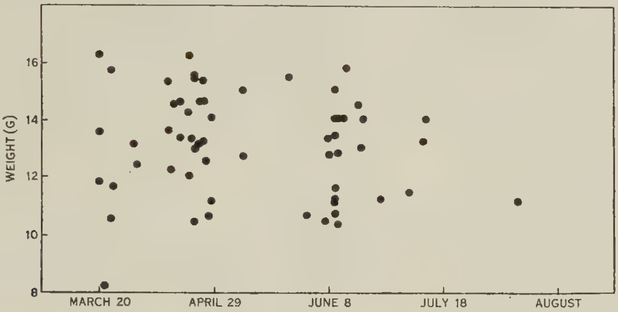

Fig. 3.—Effect of season on body weight of mice at 4 wk.

V. Analysis

Two possible environmental trends were first investigated to see what could be done to decrease environmental effects by direct corrections. These are shown in Figures 2 and 3. The effect of litter number on individual weight (Fig. 2) is not very great and it is clear that the regression, which is about 0·12 g decrease in weight for each increase of one in litter number, is not going to remove much of the variance of weight. A seasonal trend of some sort seems to be indicated in Figure 3 but the data do not group themselves closely round

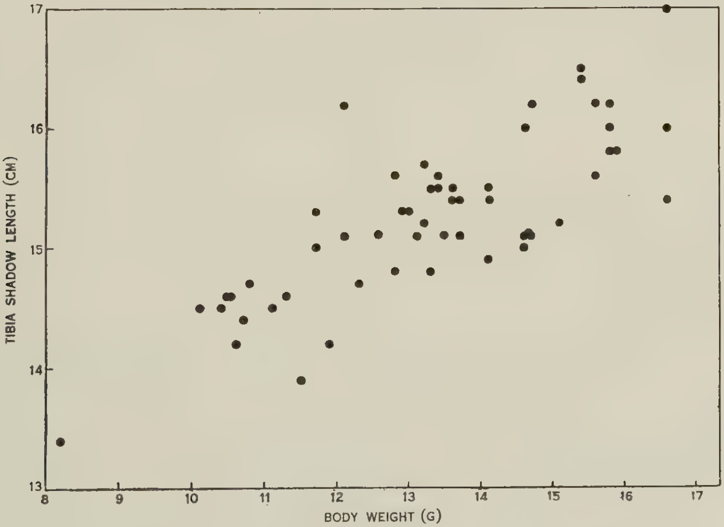

Fig. 4.—Correlation of tibia shadow length and body weight in mice at 4 wk.

any line, and the variance removed by correcting for season would be very small indeed in this instance. The effects of these two environments on tibia length are similar and have not been shown.

TABLE 2

SUMS OF SQUARES AND CROSS PRODUCTS OF BODY WEIGHT AND TIBIA LENGTH AT 4 WK

Source	D.f.	Sums of Squares, Body Weight	Sum of Cross Products	Sums of Squares, Tibia Length
Between sires	14	287·9050	57·3800	23·8480
Between litters of the same sire	42	577·7697	184·0134	91·5735
Between individuals of the same litter	270	454·8000	74·2344	33·6846

The plot of tibia length against weight (Fig. 4) shows a much closer clustering round the regression line. The correlation is about 0·7 and the regression line will account for some 50 per cent. of the total variance. This is in marked contrast to the effect of season and litter number and, provided the correlation is largely an environmental one, it will remove a great deal more environmentally caused variance than the other two put together. It is clear that a considerable search through possible environmental effects would have to be made before as much variance could be accounted for as is removed by the regression line of weight on tibia length and vice versa. This search has not been made here because factors which might have influenced body weight and tibia length, such as temperature and disease, were not recorded in sufficient detail.

TABLE 3

REGRESSIONS OF BODY WEIGHT ON TIBIA LENGTH AND TIBIA LENGTH ON BODY WEIGHT

Source	Regression	
	Body Weight on Tibia Length	Tibia Length on Body Weight
Between sires	2·405	0·199
Between litters of the same sire	2·010	0·319
Between individuals of the same litter	2·204	0·163

The sums of squared deviations and cross products of tibia length and body weight are set out in Table 2. The regressions derived from them are set out in Table 3. The regression of body weight on tibia length is the same, no matter what the source of variation. The change in body weight accompanying a unit change in tibia length is the same whether the comparison is between individuals of a litter, litters of a sire, or different sire progenies. This indicates either that the causes of the differences in tibia length are all of the same kind (e.g. environmental), no matter what the source of variance, or that changes in tibia length, whether their cause is genetic or environmental, are always accompanied by the same change in weight. The analysis of the components of variance in Table 4 shows that the former is the true explanation. There is no detectable genetic component of variance of tibia length so that an overwhelming portion of the variance will be environmental no matter what the source.

TABLE 4

ANALYSIS OF VARIANCE OF BODY WEIGHT AND TIBIA LENGTH

BODY WEIGHT

Source	Uncorrected			Corrected		
	D.f.	Mean Sq.	Component	D.f.	Mean Sq.	Component
Between sires	14	20·5646	0·3137	13	11·5343	0·2977
Between litters of same sire	42	13·7564	2·1094	41	5·0732	0·6897
Between individuals of same litter	270	1·6844	1·6844	269	1·1260	1·1260
Heritability			0·3055			0·5634

TIBIA LENGTH

Source	Uncorrected			Corrected		
	D.f.	Mean Sq.	Component	D.f.	Mean Sq.	Component
Between sires	14	1·7034	—	13	0·9548	0·0069
Between litters of the same sire	42	2·1803	0·3592	41	0·8041	0·1265
Between individuals of same litter	270	0·1248	0·1248	269	0·0802	0·0802
Heritability						0·1292

The regression of tibia length on weight is different according to the source of variation. The steepest regression is of tibia length on weight of litter. Now the genetic variance will be divided so that one-half is between individuals of a litter, one-quarter between sires, and one-quarter between dams. In this experiment between dams is the same as between litters. As the genetic variance between dam and sire should be equal, obviously the differences between litters are mostly environmental and the environmental component is much larger between dams than it is between sires or between individuals of a litter.

Where the genetic component is increased in importance relative to the non-genetic component, as it is between sires and between individuals of a litter, the regression is less, suggesting that if there is a genetic correlation between the two characters it is considerably smaller than the environmental one. The component of covariance due to differences between sires is $-0 \cdot 0003$ which, despite the absence of a component of variance in tibia length due to sire, suggests that there is no genetic correlation.

The analysis of variance shows that heritability for weight is about 0·3, assuming that all the sire component is genetic and that it includes one-quarter of the total genetic variance. There is no component from which heritability of tibia length can be measured.

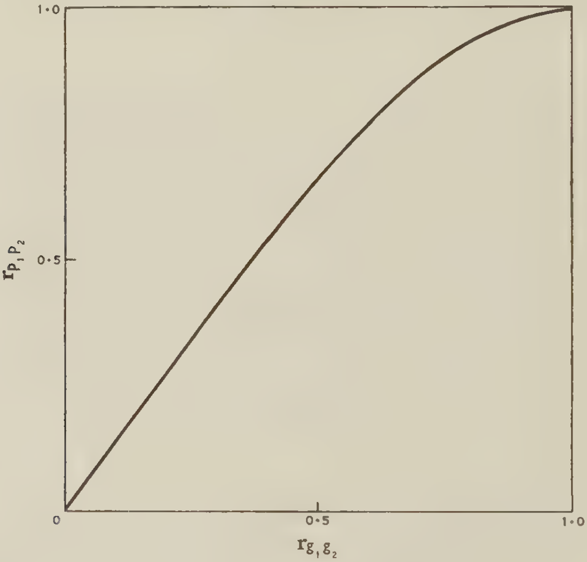

Fig. 5.—Values of r_p for different values of r_G at which $\triangle G$ is unchanged when the correction $I = X_1 - bX_2$ is applied.

After the figures were corrected for the regression of weight on tibia length and tibia length on weight the analysis was repeated. It is shown in Table 4 under the heading "corrected." In every case the regression calculated for the class corrected was used. Correction reduces the variance of both characters by about one-half, the greatest reduction being of the variance between litters. This is particularly noticeable of tibia length. The component of variance due to differences between individuals of a litter is slightly lower after correction than before, the litter component is reduced to a third and the sire component practically unchanged for weight, but now just positive for tibia length. Heritability is increased to 0·5634 for weight and about 0·1 (still a very unreliable figure) for tibia length.

The mean scores of the progeny of the 15 sires have been computed on the corrected and uncorrected basis to show what difference in practice correction would make, supposing the best five sires were selected in each case. It will be seen that the order of merit is quite considerably altered in both characters (Table 5).

TABLE 5

MEAN WEIGHT OF SIRE PROGENIES ARRANGED IN ORDER OF MERIT, CORRECTED AND UNCORRECTED

Weight					Tibia Length				
Uncorrected		Corrected			Uncorrected		Corrected		
(*a*)	15·15	(*d*)	14·33	(4)	(*b*)	15·84	(*p*)	15·60	(5)
(*b*)	14·56	(*c*)	14·02	(3)	(*a*)	15·80	(*b*)	15·58	(1)
(*c*)	14·24	(*f*)	13·93	(6)	(*g*)	15·47	(*g*)	15·48	(3)
(*d*)	14·23	(*j*)	13·75	(9)	(*e*)	15·43	(*a*)	15·42	(2)
(*e*)	14·02	(*a*)	13·73	(1)	(*p*)	15·33	(*o*)	15·34	(9)
(*f*)	13·79	(*e*)	13·49	(5)	(*c*)	15·30	(*e*)	15·28	(4)
(*g*)	13·19	(*h*)	13·36	(8)	(*d*)	15·17	(*m*)	15·26	(12)
(*h*)	13·02	(*b*)	13·04	(2)	(*f*)	15·15	(*l*)	15·21	(10)
(*j*)	12·98	(*k*)	13·04	(10)	(*o*)	15·11	(*k*)	15·19	(11)
(*k*)	12·75	(*l*)	12·94	(11)	(*l*)	15·10	(*h*)	15·12	(13)
(*l*)	12·68	(*n*)	12·92	(13)	(*k*)	15·09	(*j*)	15·11	(14)
(*m*)	12·40	(*m*)	12·69	(12)	(*m*)	15·09	(*c*)	15·10	(6)
(*n*)	12·15	(*g*)	12·56	(7)	(*h*)	15·07	(*f*)	15·04	(8)
(*o*)	12·09	(*o*)	12·33	(14)	(*j*)	14·89	(*d*)	14·97	(7)
(*p*)	11·90	(*p*)	11·61	(15)	(*n*)	14·89	(*n*)	14·94	(15)

Letters in brackets identify the sires.
Figures in brackets indicate the ranking of the sire before correcting.

VI. DISCUSSION

The foregoing exercise shows that where a suitable character is available it is far simpler and more effective to discount environmental effects by correcting one character for its regression on another than by searching out the separate environmental trends. A suitable character is one which has no economic value, is strongly correlated phenotypically with the desired character, but has little or no genetic correlation with it. It is worth noting that even if there is a genetic correlation there are two general situations in which the correction is still worth applying; first, when the phenotypic correlation is sufficiently greater than the genetic one, and second, where the second character is one which it is desired to keep reasonably constant. For example, in Denmark, selection for milk yield and fat yield has been accompanied by an increase in body size, to a point where some farmers are faced with rebuilding their cow sheds, or changing their breed. It is believed that the Danish Red by Jersey cross has gained in popularity for this reason. Under such circumstances one might be willing to sacrifice increase in yield which accompanies increase in size to save capital expenditure. But where a genetic correlation is known to exist and has been measured, the full index should be used to determine S_2.

There is one possible danger in using this method of correction as a basis of selection, which is that under normal methods animals sensitive to bad environments will be selected against, but after correcting for environmental trends, although there will probably be some selection against sensitivity to the environment, it will not be nearly so strong.

VII. ACKNOWLEDGMENTS

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THE EFFECT OF SOME SEMINAL CONSTITUENTS AND RELATED SUBSTANCES ON DILUTED MAMMALIAN SPERMATOZOA

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Summary

The effect has been studied of a number of seminal constituents and related substances on the motility of diluted ram, bull, rabbit, and human spermatozoa. All tests were made with a fructose phosphate diluent over a 4-hr period at room temperature.

Trace concentrations ($< 0 \cdot 20$ mg/100 ml) of copper, cobalt, manganese, iron, and zinc had little effect on ram, bull, or rabbit spermatozoa, except that copper depressed the motility of ram spermatozoa.

Biotin improved the viability of bull spermatozoa but had no effect on ram or rabbit spermatozoa. Thiamine, riboflavin, niacin, inositol, *p*-aminobenzoic acid, pantothenic acid, and folic acid were inactive in all species.

None of 21 amino acids had any significant beneficial action on ram, bull, or rabbit spermatozoa, and some were toxic.

Of several miscellaneous seminal constituents, cytochrome *c* and spermine phosphate improved the motility of rabbit spermatozoa but had no effect on ram or bull spermatozoa. Citric acid, choline chloride, CoI, and adenosine triphosphate were inactive and ascorbic acid toxic to the spermatozoa of all three species.

Human spermatozoa were found to be particularly susceptible to dilution and no protective action was shown by substances that were beneficial to other mammalian spermatozoa.

I. Introduction

The harmful effect of dilution on the motility of spermatozoa was first reported by Milovanov (1934*a*, 1934*b*) who found that spermatozoa could be immobilized if sufficient of 1 per cent. sodium chloride was added to semen. This was the basis for his test of the resistance of ejaculates; the index of resistance, *R*, being the volume of diluent required, divided by the volume of semen. Milovanov's observation has been confirmed for rabbit, human, bull, and ram spermatozoa and it would appear that the dilution phenomenon is of general occurrence with mammalian spermatozoa (Chang 1942; Kennedy 1947; Rao and Hart 1948; Blackshaw 1953).

The effect is not, as Milovanov supposed, due to the toxicity of sodium chloride, since it occurs with chloride-free sulphate, tartrate, and citrate diluents (Salisbury *et al.* 1943; Emmens and Swyer 1948; Cheng, Casida, and Barrett 1949; Blackshaw 1953). Tonicity changes and the dilution of seminal substrate are not important factors either, since motility is still depressed in isotonic diluents containing glucose or fructose (Emmens and Swyer 1948; Blackshaw 1953; White 1953*a*).

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Emmens and Swyer (1948) found that accessory secretion protected rabbit spermatozoa to some extent from dilution, but it was not as effective as supernatants from spermatozoa left overnight. The depression of motility at low cell concentrations would seem, therefore, to be due to both dilution of plasma material and loss of substances from the spermatozoa. Miscellaneous high-molecular-weight substances (e.g. proteins and starch) that have a protective action on diluted spermatozoa presumably act by preventing the latter (Emmens and Swyer 1948; Blackshaw 1953; White 1953*a*).

White (1953*a*, 1953*b*, 1953*c*) found that potassium increases the motility and glycolysis of repeatedly washed or moderately diluted (20×10^6/ml) ram and bull spermatozoa. This suggests that potassium is lost from the cells under these conditions. At very low spermatozoa concentrations ($< 2 \times 10^6$/ml), however, substances other than potassium must be limiting since motility in potassium-containing diluents is little or no better than in potassium-free diluent (White 1953*a*).

The effect has now been studied of a number of seminal constituents and related substances on the motility of diluted ram, bull, rabbit, and human spermatozoa and the results are presented in this paper.

II. MATERIALS AND METHODS

(*a*) *Semen*

Ram semen was obtained by electrical ejaculation (Gunn 1936); bull and rabbit semen was collected in an artificial vagina, and human semen was obtained from a sterility clinic. Only normal ejaculates of good initial motility were employed, and were used singly. Experiments with ram and rabbit semen were started immediately after collection and those with bull semen within 2 hr of collection; the bull semen was slowly cooled to about 10°C and kept at this temperature during transport to the laboratory. The human semen was between 4 and 6 hr old before use.

(*b*) *Technique*

Spermatozoal counts were made in duplicate on the neat semen using a haemocytometer; the semen was then diluted with an isotonic diluent of pH 7·0 having the following composition:

$0{\cdot}032$M $NaH_2PO_4.H_2O$, $0{\cdot}048$M $Na_2HPO_4.12H_2O$, $0{\cdot}036$M NaCl,
$0{\cdot}004$M KCl, $0{\cdot}004$M $MgCl_2$, $0{\cdot}022$M fructose.

With the exception of 500 mg/100 ml glutamic acid, which brought the pH of the diluent down to 6·0, the other substances added had no appreciable effect. Tests were made on ram and bull spermatozoa at a cell concentration of 2×10^6/ml and on rabbit and human spermatozoa at $0{\cdot}4 \times 10^6$/ml. All experiments were done with open tubes at room temperature.

Motility was scored at hourly intervals over a 4-hr period by the system of Emmens (1947). Full motility was rated as 4 and complete immotility as zero, but in presenting results the actual scores have been multiplied by 4 since quarter grades were frequently used.

(*c*) *Statistical Treatment*

The results have been subjected to standard analyses of variance (cf. Snedecor 1946) with isolation of sums of squares attributable to differences between ejaculates and treatments. The total motility score (× 4) has been used as unit observation and the interaction mean square as error term. Differences between ejaculates are often very significant, so that the accuracy of comparisons is much improved by analysis in this way.

F values have been given in the text. When, as is sometimes the case, motility falls rapidly, treatment variances are probably not completely independent of the level of response. Since, however, an effect has only been judged significant when the probability of its being due to chance is less than 1 in 100 this is of little consequence.

(*d*) *Chemicals*

AR sulphate salts of the minerals were used. The L or DL amino acids were B.D.H. laboratory reagents; except that ornithine, arginine, leucine, isoleucine, valine, cystine, and di-iodotyrosine were supplied by Light & Co. Ltd. Glycine was supplied by May and Baker Ltd.

The vitamins and other substances were obtained from the following sources:

Nicotinamide and folic acid—Roche Products Ltd.
Para-aminobenzoic acid, riboflavin, and pyridoxide—Andrews Laboratories.
Calcium pantothenate, thiamine hydrochloride, and vitamin B_{12} (saline solution)—General Biochemicals.
Inositol—Difco Laboratories.
Biotin (in phosphate buffer)—British Drug Houses Ltd.
Ascorbic acid—British Drug Houses Ltd. and Colonial Sugar Refinery Co.
Adenosine triphosphate (free acid; total $P = 14 \cdot 6\%$, $64 \cdot 5\%$ hydrolysable; $N = 11 \cdot 0\%$; $N/P = 0 \cdot 75$); and CoI (61·4% DPN)—Schwarz Laboratories.
Cytochrome *c* (0·34% Fe)—National Biochemical Corp.
Spermine phosphate and choline chloride—Light & Co. Ltd.
Crystalline bovine albumin—Armour Laboratories.

III. Results

(*a*) *Ram, Bull, and Rabbit Spermatozoa*

The nature of the dilution effect is illustrated by the results of preliminary tests (Table 1) in which motility was scored at high and low spermatozoa concentration for each species. Not only was the motility depressed initially by dilution but it fell off more rapidly at the lower cell concentrations. The *F* values for the analyses of variance are 70·0, 30·5, and 84·8 for the ram, bull, and rabbit respectively and $P < 0 \cdot 01$ in each case.

In subsequent tests the semen was diluted for convenience in two stages. Motility in the neat semen and at the intermediate cell concentration has not been recorded but was invariably better than at the final dilution to which the figures in subsequent tables refer. It may be noted that the susceptibility to dilution of ejaculates from the same species often varied considerably although initial motility in the neat semen was good in each.

Table 1

EFFECT OF DILUTION ON THE HOURLY MOTILITY SCORE (× 4) OF RAM, BULL, AND RABBIT SPERMATOZOA. EACH VALUE IS THE MEAN OF SIX EJACULATES

Species	Sperm Concentration ($\times 10^{-6}$/ml)	Hours					Total
		0	1	2	3	4	
Ram	200·0	15	15	15	15	15	75
	2·0	10	6	6	3	1	26*
Bull	200·0	14	13	12	11	8	57
	2·0	8	6	5	4	3	26*
Rabbit	20·0	14	13	12	12	11	62
	0·4	8	6	4	3	2	23*

*Significantly < score at higher cell concentration, $P < 0 \cdot 01$.

(i) *Trace Elements.*—Mammalian semen is known to contain small amounts of copper, iron, and zinc (Zittle and Zitin 1942; Mann 1945), and the report of Lardy, Phillips, and Rupel (1942) suggests that manganese might be important for the maintenance of the motility of bull spermatozoa. The addition to the diluent of these elements in physiological concentrations (copper, cobalt, manganese = 0·05 mg/100 ml; iron, zinc = 0·20 mg/100 ml) did not, however, significantly improve the motility of diluted ram, bull, or rabbit spermatozoa. The results of tests on four ejaculates from each species are given in Table 2. Copper, it may be noted, significantly depressed the motility of diluted ram spermatozoa ($F = 12 \cdot 0$, $P < 0 \cdot 01$).

Table 2

TOTAL MOTILITY SCORE (× 4) OF DILUTED RAM, BULL, AND RABBIT SPERMATOZOA IN THE PRESENCE OF HEAVY METAL IONS. EACH VALUE IS THE MEAN OF FOUR EJACULATES

Species	Control	Copper	Cobalt	Iron	Manganese	Zinc
Ram	38	27*	41	43	43	42
Bull	19	20	23	18	23	20
Rabbit	9	7	8	8	8	7

*Significantly toxic, $P < 0 \cdot 01$.

TABLE 3

TOTAL MOTILITY SCORE (× 4) OF DILUTED RAM, BULL, AND RABBIT SPERMATOZOA IN THE PRESENCE OF THE B VITAMINS. EACH VALUE IS THE MEAN OF FOUR EJACULATES

Species	Control	Thiamine (0·5 mg/ 100 ml)	Riboflavin (0·5 mg/ 100 ml)	Niacin (0·5 mg/ 100 ml)	Inositol (0·5 mg/ 100 ml)	PAB (0·5 mg/ 100 ml)	Pantothenic Acid (0·5 mg/ 100 ml)	Pyridoxine (0·5 mg/ 100 ml)	Folic Acid (0·5 mg/ 100 ml)	Biotin (25 μg/ 100 ml)	Vitamin B_{12} (40 μg/ 100 ml)
Ram	38	40	43	34	41	41	43	44	39	37	42
Bull	10	12	10	11	11	10	11	8	9	36†	11
Rabbit	34	36	37	34	35	35	29	27	34	36	34

† Significantly beneficial, $P<0\cdot01$.

(ii) *Vitamin B Complex.*—Thiamine, riboflavin, niacin, and pantothenic acids are present in bull semen (Lardy and Phillips 1941; VanDenmark and Salisbury 1944), and inositol has recently been found to be a major constituent of boar semen (Mann 1951*a*). It was of interest, therefore, to see if these or other members of the vitamin B complex were involved in the dilution effect, particularly as VanDenmark and Salisbury (1944) claimed that the thiamine, riboflavin, and niacin levels of bull semen are correlated with the initial motility of the spermatozoa. Thiamine, riboflavin, niacin, inositol, *p*-aminobenzoic acid, pantothenic acid, pyridoxine, and folic acid were each tried at a concentration of 0 5 mg/100 ml. Biotin and vitamin B_{12} had to be used in lower concentrations (25 and 40 μg/100 ml respectively) because they were supplied as solutions in ampoules.

The results for four ejaculates from each species are set out in Table 3. Biotin increased the viability of bull spermatozoa ($F = 108{\cdot}4$) but did not significantly influence the motility of ram or rabbit spermatozoa. The other members of the vitamin B complex had little or no effect on the spermatozoa of any of the three species.

Confirmatory tests on six other bull ejaculates (Table 4) showed that biotin significantly improved motility at 5 μg/100 ml ($F = 18{\cdot}8$) and 10 μg/100 ml ($F = 54{\cdot}5$).

TABLE 4

EFFECT OF BIOTIN ON THE TOTAL MOTILITY SCORE (× 4) OF DILUTED BULL SPERMATOZOA

Ejaculate	Biotin (μg/100 ml)		
	0	5	10
1	12	46	62
2	13	36	63
3	7	55	72
4	26	38	76
5	28	51	53
6	33	47	55
Mean	20	46*	64*

*Significantly beneficial, $P < 0{\cdot}01$.

(iii) *Amino Acids.*—Recent reports indicate that free amino acids are present in bull semen and accessory fluid (Gassner and Hopwood 1952; Larson and Salisbury 1953). A number have therefore been tested on diluted bull, ram, and rabbit semen. Twenty-one amino acids were each tried at two concentrations (500 and 50 mg/100 ml). The tests were done on four ejaculates from each species at both concentrations.

Table 5 shows that none of the amino acids at either concentration improved the motility of diluted ram, bull, or rabbit spermatozoa to any great extent.

On the other hand, some of the amino acids significantly depressed motility. Di-iodotyrosine at 500 mg/100 ml rapidly immobilized ram ($F = 13\cdot 5$), bull ($F = 69\cdot 4$), and rabbit spermatozoa ($F = 29\cdot 9$) and also depressed the motility of ram ($F = 21\cdot 8$) and rabbit spermatozoa ($F = 31\cdot 5$) at 50 mg/100 ml. Tyrosine was toxic to ram spermatozoa at 500 ($F = 13\cdot 9$) and 50 mg/100 ml ($F = 12\cdot 1$) and to rabbit spermatozoa at the higher concentration ($F = 9\cdot 9$).

TABLE 5

TOTAL MOTILITY SCORES (× 4) OF DILUTED RAM, BULL, AND RABBIT SPERMATOZOA IN THE PRESENCE OF AMINO ACIDS AND GLUTATHIONE. EACH VALUE IS THE MEAN OF FOUR EJACULATES

Amino Acids	Ram		Bull		Rabbit	
	Amino Acid Concentration		Amino Acid Concentration		Amino Acid Concentration	
	500 mg/ 100 ml	50 mg/ 100 ml	500 mg/ 100 ml	50 mg/ 100 ml	500 mg/ 100 ml	50 mg/ 100 ml
Nil	22	42	31	23	25	33
Glycine	25	40	34	25	29	36
Alanine	18	42	36	25	31	31
Valine	25	36	37	25	24	29
Leucine	24	39	36	24	29	29
Isoleucine	24	38	35	23	29	29
Serine	23	36	37	25	25	34
Glutamic Acid	15	38	32	36	8*	34
Tryptophane	21	38	28	22	28	24
Proline	25	38	38	24	29	26
Histidine	23	35	36	27	20	29
Ornithine	21	39	36	19	26	30
Citrulline	29	—	36	—	29	—
Arginine	19	40	35	26	31	24
Lysine	23	38	35	21	27	30
Cysteine	3*	14*	13*	18	9*	16*
Cystine	28	37	29	24	20	26
Methionine	27	36	37	25	29	32
Glutathione	6*	32*	19*	25	11*	22*
Phenylalanine	15	34	28	23	24	34
Tyrosine	4*	31*	27	19	11*	37
Di-iodotyrosine	4*	27*	6*	20	1*	12*

*Significantly toxic, $P<0\cdot 01$.

Cysteine depressed the motility of ram ($F = 15\cdot 0$), bull ($F = 38\cdot 1$), and rabbit spermatozoa ($F = 13\cdot 3$) at 500 mg/100 ml and was also toxic to ram ($F = 75\cdot 7$) and rabbit ($F = 21\cdot 3$) spermatozoa at 50 mg/100 ml. Glutathione, which is a cysteine derivative, was similarly toxic to ram ($F = 10\cdot 7$), bull ($F = 16\cdot 7$), and rabbit spermatozoa ($F = 9\cdot 6$) at the higher concentration and to ram ($F = 10\cdot 0$) and rabbit spermatozoa ($F = 9\cdot 0$) at 50 mg/100 ml.

Glutamic acid at 500 mg/100 ml also proved harmful to rabbit spermatozoa ($F = 14{\cdot}1$). This effect may, however, have been due to the fall in pH of the diluent caused by the glutamic acid. It should be noted that these effects may not always be seen if motility is too greatly depressed by dilution.

TABLE 6

TOTAL MOTILITY SCORE (× 4) OF DILUTED RAM, BULL, AND RABBIT SPERMATOZOA IN THE PRESENCE OF SOME MISCELLANEOUS SEMINAL CONSTITUENTS. EACH VALUE IS THE MEAN OF FOUR EJACULATES

Species	Experiment	Control	Cytochrome *C* (2·5 mg/100 ml)	Coenzyme I (2·5 mg/100 ml)	Adenosine Triphosphate (50 mg/100 ml)	Spermine Phosphate (50 mg/100 ml)	Citric Acid (25 mg/100 ml)	Choline Chloride (250 mg/100 ml)	Ascorbic Acid (10 mg/100 ml)
Ram	1	41	45	48	36	40	—	—	—
	2	44	—	—	—	—	40	40	—
	3	27	—	—	—	—	—	—	10*
Bull	1	18	20	19	23	17	19	21	—
	2	28	—	—	—	—	—	—	15
Rabbit	1	19	43†	29	24	31†	—	—	—
	2	27	—	—	—	—	26	29	9*

†Significantly beneficial, $P<0{\cdot}01$. *Significantly toxic, $P<0{\cdot}01$.

(iv) *Miscellaneous Seminal Constituents.*—The substances tested were citric acid (25 mg/100 ml), choline chloride (250 mg/100 ml), spermine phosphate (50 mg/100 ml), cytochrome *c* (2·5 mg/100 ml), CoI (2·5 mg/100 ml), adenosine triphosphate (50 mg/100 ml), and ascorbic acid (10 mg/100 ml). Each of these has been found in the semen of one or more mammalian species (see Mann 1949) and in choosing the concentrations, the amounts reported in neat semen have, where possible, been taken as a guide. The results for four ram, bull, and rabbit ejaculates are given in Table 6. None of the substances improved the motility of diluted ram or bull spermatozoa to any great extent. Cytochrome *c* ($F = 44{\cdot}2$) and spermine phosphate ($F = 11{\cdot}5$), however, were significantly beneficial to diluted rabbit spermatozoa.

Confirmatory tests on four rabbit ejaculates at three concentrations of cytochrome *c* (0·1, 1·0, and 10·0 mg/100 ml)and spermine phosphate (5·0, 50·0, and 500·0 mg/100 ml) are shown in Table 7. Cytochrome *c* was significantly beneficial at 10 ($F = 16{\cdot}6$) and 1·0 mg/100 ml ($F = 13{\cdot}7$) and spermine phosphate improved viability at 500 ($F = 11{\cdot}4$) and 50 mg/100 ml ($F = 8{\cdot}4$).

Ascorbic acid significantly depressed the motility of diluted ram ($F = 61{\cdot}2$) and rabbit spermatozoa ($F = 41{\cdot}6$) and had a similar effect on bull spermatozoa, although it is not quite significant at the 0·01 probability level.

(*b*) *Human Spermatozoa*

Limited tests were made on human spermatozoa, using biotin, cytochrome *c*, spermine phosphate, CoI, glycine, choline chloride, and the high-molecular-

TABLE 7

EFFECT OF CYTOCHROME *c* AND SPERMINE PHOSPHATE ON THE TOTAL MOTILITY SCORE (× 4) OF DILUTED RABBIT SPERMATOZOA

Ejaculate	Control	Cytochrome *c* (mg/100 ml)			Spermine Phosphate (mg/100 ml)		
		0·1	1·0	10·0	5·0	50·0	500·0
1	3	10	37	24	19	21	32
2	23	22	29	47	41	45	46
3	4	13	38	37	22	20	13
4	9	8	14	18	8	15	20
Mean	10	13	28†	32†	23	25†	28†

†Significantly beneficial, $P<0{\cdot}01$.

weight substances previously shown to improve the motility of diluted rabbit, bull, and ram spermatozoa (Emmens and Swyer 1948; White 1953*a*; Blackshaw 1953). Table 8 gives the results of four ejaculates. It is clear that human spermatozoa are particularly susceptible to dilution and that none of the compounds tried was of much value in maintaining motility.

TABLE 8

TOTAL MOTILITY SCORE (× 4) OF HUMAN SPERMATOZOA IN THE PRESENCE OF SEMINAL CONSTITUENTS AND MISCELLANEOUS SUBSTANCES

Treatment	Sperm Concentration (× 10^{-6}/ml)	Mean of Four Ejaculates
Control	20·0	54
Control	0·4	4
Cytochrome *c* (2·5 mg/100 ml)	0·4	4
Coenzyme I (2·5 mg/100 ml)	0·4	4
Spermine phosphate (50 mg/100 ml)	0·4	3
Choline chloride (250 mg/100 ml)	0·4	4
Biotin (10 μg/100 ml)	0·4	7
Glycine (500 mg/100 ml)	0·4	5
Plasma albumin (250 mg/100 ml)	0·4	6
Egg albumin (250 mg/100 ml)	0·4	4
Glycogen (250 mg/100 ml)	0·4	4
Starch (250 mg/100 ml)	0·4	5

IV. DISCUSSION

These tests (Tables 5 and 6) suggest that loss of biotin might be a factor in the harmful effect of dilution on bull spermatozoa. Egg yolk, which is widely used as an extender for bull semen, is a rich source of biotin and is known to protect bull spermatozoa to some extent from dilution (Cheng, Casida, and Barrett 1949).

The two substances that improved the viability of rabbit spermatozoa in these tests were cytochrome *c* and spermine phosphate. Mann (1951*b*) has shown that cytochrome *c* is leached out of mammalian spermatozoa on storage and spermine phosphate is believed to be contributed to semen by the prostate (Rosenheim 1924; Harrison 1931, 1933; Bolliger 1935). These substances could therefore be the active compounds of the spermatozoa and accessory fluid, respectively, referred to in the introduction.

No clue, however, has been obtained as to the factors that might be involved in the deleterious effect of dilution on ram and human spermatozoa.

Knoop and Krauss (1944) claimed that 1 per cent. glycine or proline improved the efficacy of egg yolk as an extender for bull semen and more recently it has been reported that glycine increases the life span of fowl and bull spermatozoa diluted in saline and balanced salt solutions (Lorenz and Tyler 1951; Tyler and Tanabe 1952). The latter workers, however, believe that the beneficial effect of glycine in their experiments was due to the binding of toxic trace elements in the diluent. Glass-distilled water was used in the present experiments, which may account for the inactivity of glycine. Tosic and Walton (1950) reported that tyrosine, phenylalanine, and tryptophane are toxic to bull spermatozoa on shaking in air—an effect which they attributed to the formation of hydrogen peroxide. Macleod (1951) has also found that cysteine, but not glutathione, depresses the motility of human spermatozoa and suggested that its action is due to the oxidation of essential thiol groups in the spermatozoa.

The toxic action of ascorbic acid in these experiments is rather surprising and was apparently not due to an impurity in the chemical as similar results were obtained with two preparations from independent sources. However, the concentrations used were similar to those reported in neat semen (Phillips *et al.* 1940; Berg, Huggins, and Hodges 1941), and diluted spermatozoa would not normally be in contact with such amounts.

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THE REACTION OF THE ZONA PELLUCIDA TO SPERM PENETRATION

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Summary

The observations described concern the number of sperms seen within the eggs of the rat, mouse, rabbit, sheep, and dog.

A frequency distribution is presented for the eggs of 251 rats and 20 mice, according to the number of sperms that they were found to contain.

Considering the high proportion of penetrated eggs in both the rats and the mice, the number of eggs that contained more than one sperm (about 20 per cent.) was much smaller than would be expected if the distribution were controlled by chance alone. This is interpreted to mean that the penetrability of the zona pellucida to sperms decreases after the entry of the first sperm. Such a reaction has not previously been demonstrated in the zona pellucida. In eggs that contained two sperms, the points of entry through the zona were seen much more frequently in opposite hemispheres than in the same hemisphere, an observation that suggests the zona reaction is a propagated change. It is estimated that the mean time the reaction takes to reach completion is not less than 10 min or more than 1½-2 hr.

In the rabbit the penetrability of the zona pellucida is not influenced by sperm entry. The mean number of sperms in the perivitelline space of the rabbit egg increased from 0·2 10 hr after coitus to 61·8 16 hr after coitus; thereafter there was no significant change.

No sperms were detected in the perivitelline space of 12 fertilized sheep eggs or in 12 fertilized dog eggs, although numerous sperms adhered to the surface of the zona pellucida. The zona pellucida of both sheep and dog eggs apparently reacts to the entry of the fertilizing sperm in such a way as to preclude further penetration.

Published reports on the number of sperms within the eggs of various mammals are reviewed. It is concluded that, except in the eggs of the rabbit and perhaps the mole, the zona pellucida of mammals reacts to sperm penetration in such a way that few, if any, extra sperms can enter the eggs. The importance of this reaction in relation to fertility is discussed.

I. Introduction

The zona pellucida of the mammalian egg is an apparently structureless membrane that surrounds the vitellus but is separated from it by the perivitelline space. It is mucoprotein in nature (Braden 1952). The zona persists during the cleavage of the egg in the fallopian tube but disappears shortly after the egg enters the uterus. In spite of its temporary and apparently passive function as a protective membrane, the zona pellucida is nevertheless capable of exhibit-

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ing changes in state which are reflected in variations in its penetrability to sperms. It has been found recently that, in the rat, sperms do not enter the eggs immediately after ovulation. Evidently the egg membranes must first undergo some change, which represents perhaps a terminal phase in maturation (Austin and Braden 1954*b*). When this change has been effected, a sperm may readily traverse the zona at any time during the subsequent 12 hr. However, data have now been obtained indicating that, in many mammals, the entry of the first sperm into the egg initiates a reaction in the zona the effect of which is to preclude the entry of further sperms. This reaction resembles, in its consequence, the change that occurs in the vitellus, the so-called "block to polyspermy." Evidence for the occurrence of such a reaction in the zona, and an assessment of its speed, are the subjects of the present communication.

II. Methods

The experimental animals included rats of albino and hooded strains, albino mice, cross-bred rabbits, Merino ewes, and mongrel bitches.

The rats were mated before, during, or after ovulation, which has been shown to occur between midnight and 4 a.m. (Austin and Braden 1954*b*). "Normally" mated rats were those that were left with males overnight from 4 p.m. Coitus generally occurred in the early evening. Rats that mated were recognized by the presence of copulation plugs when examined the next morning. Other groups of rats were placed with males at 10 p.m., 3 a.m., or 8 a.m. and were permitted 1 hr for copulation. Normally mated rats and those mated at 10 p.m. and 3 a.m. were killed between 11 a.m. and 4 p.m. on the day of ovulation. Rats mated at 8 a.m. were killed at 10 a.m. on the following day. The eggs were recovered from the fallopian tubes by dissection under normal saline solution and were examined with the phase-contrast microscope.

Mice were left with males overnight and those with copulation plugs next morning were killed during the course of the day.

Rabbits were permitted to copulate, each female with two males, and killed 10, 13, 16, and 20 hr later. The eggs were recovered by flushing the tubes with normal saline solution and were fixed for several hours in 10 per cent. acetic-alcohol. They were embedded by Minchin's albumen method (Gatenby and Beams 1950), cut in 7-μ sections, and stained by the Feulgen technique. The sperm heads in the eggs could thus be readily recognized and counted.

The ewes were run with a raddled ram and were inspected each night and morning for signs of mating. Those that mated were killed 18-36 hr later. The eggs were recovered by the same method as was used for the rabbit and were examined in the fresh state.

The genital tracts of two bitches were removed by laparotomy 3-4 days after coitus. The eggs were recovered in the same way as for the rabbit. Eight eggs were examined in the fresh state; the remainder were fixed in 10 per cent. formol-saline and then embedded, sectioned, and stained in the same way as were the rabbit eggs.

III. Observations

(a) *Frequency Distribution of Rat and Mouse Eggs According to the Number of Penetrating Sperms*

From 87 normally mated rats, 826 eggs were obtained; 810 (98·1 per cent.) had been penetrated by sperms, most of them (76·1 per cent.) by one sperm only. The frequency with which eggs were seen to contain more than one sperm diminished progressively with larger numbers of penetrating sperms. Thus 135 eggs (16·7 per cent.) had two sperms, 25 eggs (3·1 per cent.) had three sperms, and 12 eggs (1·5 per cent.) had four sperms. Only 10 eggs were found to contain five or more sperms; two eggs were exceptional for they had about 25 and 50-60 sperms respectively, and were degenerate in appearance (Plate 1, Fig. 6). Ten of the eggs with two or more sperms were polyspermic, i.e. two sperms had entered the vitellus; all the other sperms that were present in excess of one for each egg were in the perivitelline space and had not penetrated the vitellus (Table 1).

Similar observations were made on rats mated at 10-11 p.m., 3-4 a.m., and 8-10 a.m., and similar frequency distributions were apparent among the eggs recovered. The only notable differences were that both the number of eggs containing two or more sperms, and the number of polyspermic eggs, increased with the later times of mating (Table 1). From these rats, four exceptional eggs were seen that contained 10, 12, 16, and 18 sperms respectively; the eggs with 16 and 18 sperms were clearly degenerate.

The pattern of the frequency distributions seemed to show that the proportion of eggs with only one sperm was higher than would be expected if the number of sperms that entered an egg was controlled by chance alone. This possibility was investigated by selecting from the normally mated rats a group that had been killed in the course of a single experiment, and arranging the numbers of eggs from these animals in the form of a frequency distribution based upon the number of penetrating sperms. Thus from 10 rats, 91 eggs were recovered, of which five were unpenetrated, 68 contained one sperm, 15 contained two sperms, one contained three sperms, and two contained four sperms. There were no eggs with five or more sperms. A theoretical or expected distribution was then calculated for the same total number of eggs on the assumption that chance alone controlled the number of sperms that entered the eggs (Table 2). The two distributions, the observed and the expected, differed widely from each other. In both, the largest group was made up of the eggs that contained only one sperm, but the number of eggs in this group was much lower in the expected series (33·1) than in the observed (68). On the other hand, the number of unpenetrated eggs, and the numbers of eggs containing two or more sperms, were much higher in the theoretical distribution. Clearly, a much greater proportion of eggs was observed with only one sperm than would be expected if chance alone controlled the number of sperms which penetrated the zona. This is interpreted to mean that, as a result of the penetration of the first sperm, some change occurs in the zona pellucida that tends to prevent the penetration of later sperms. From

TABLE 1

FREQUENCY DISTRIBUTION OF RAT AND MOUSE EGGS THAT CONTAINED VARIOUS NUMBERS OF SPERMS WITHIN THE ZONA PELLUCIDA

Species	Time of Mating	Number of Animals	Total Eggs	Unpenetrated Eggs	Eggs Penetrated by *i* Sperms									Polyspermic Eggs*	Total Penetrated Eggs
					i = 1	2	3	4	5	6	7	8	>8		
Rats	Normal	87	826	16	628	135	25	12	3	0	4	1	2 (25, 50-60)	10	810
Rats	10-11 p.m.	69	656	9	482	126	21	13	2	1	1	0	1 (10)	18	647
Rats	3-4 a.m.	22	235	8	158	45	13	5	3	2	1	0	0	19	227
Rats	8-10 a.m.	73	649	55	396	128	42	15	6	2	2	0	3 (12, 16, 18)	51	594
Mice	Normal	20	178	9	138	27	2	1	0	1	0	0	0	2	169

* The polyspermic eggs are distributed through the other columns according to the total number of sperms within the zona pellucida.

the data in Table 1, it will be seen that similar conclusions may be drawn, not only for the complete results from rats mated at the normal time, but also from those mated at later periods.

The expected distribution was determined in the following way. Consider any one tube. If the assumption is made that the chances of sperm penetration into an egg are unaffected by the entry of the first sperm, then the penetrating sperms may be supposed to be randomly distributed amongst the eggs. Let there be s penetrating sperms and n eggs. If each egg has the same chance of being penetrated, this chance will be $1/n$, and the probability of any one egg having i sperms in it will be given by the binomial term

$$\binom{s}{i}\left(1-\frac{1}{n}\right)^{s-i}\left(\frac{1}{n}\right)^{i} \qquad (0 \leqslant i \leqslant s).$$

Hence the expected number of eggs with i sperms will be

$$\binom{s}{i}\left(1-\frac{1}{n}\right)^{s-i}\left(\frac{1}{n}\right)^{i-1}.$$

From this expression it is possible to obtain expected frequencies corresponding to the observed frequencies. Since, however, both s and n are small for any one fallopian tube, it is necessary to pool the results from a number of tubes, by adding the expected numbers of eggs with i sperms, and in a similar manner the corresponding observed numbers. The results are set out in Table 2.

TABLE 2

OBSERVED AND EXPECTED FREQUENCIES OF RAT AND MOUSE EGGS CONTAINING VARIOUS NUMBERS OF SPERMS

Species	Frequency	Number of Eggs Containing i Sperms						Total Eggs
		$i=$ 0	1	2	3	4	$\geqslant 5$	
Rats	Observed	5	68	15	1	2	0	91
	Expected	26·8	33·1	20·7	7·8	2·1	0·6	91·1
Mice	Observed	3	42	10	0	1	0	56
	Expected	15·3	22·4	12·8	4·4	1·0	0·2	56·1

So far, the assumption has been made that eggs do not differ in their penetrability. It is reasonable to suppose that this is not so, as a few eggs, for example, were found with very large numbers of sperms. However, the effect of allowing for differences in penetrability will be simply to increase both ends of the theoretical distribution, i.e. it would increase the numbers of eggs expected to have either no sperms or more than four sperms. This further enlarges the discrepancy between observed and expected values. The effect as regards the zero class ($i=0$) is discussed in some detail by Stevens (1937).

From similar considerations as those just described for the rats, it can be shown that in mice also the observed frequency distribution of eggs according to the number of sperms they contain differs significantly from the expected distribution. Twenty mated female mice yielded 178 eggs, of which 94·9 per cent. were penetrated, 82·6 per cent. by only one sperm (Table 1). Two of the eggs (1·2 per cent.) were polyspermic. The observed and expected distributions of mouse eggs are set forth in Table 2. The results are interpreted to mean that the zona pellucida of the mouse egg, like that of the rat egg, becomes less penetrable shortly after the entry of the first sperm.

(*b*) *Disposition of Points of Entry of Sperms that have Passed through the Zona Pellucida in the Rat*

Confirmatory evidence for the occurrence of a zona reaction was sought by noting the disposition of the small slits or potential holes made by sperms passing through the zona. The appearance of these slits has been described by Austin (1951), who found that they persisted at least until the cleavage of the egg. Slits were very often difficult to discern, but could usually be clearly shown by rolling the egg about so that some of the vitelline material exuded through them. Observations were confined to eggs that contained two sperms and subjective assessments by two independent observers were made of the angle subtended at the centre of the egg by the two slits. Sometimes one or both slits could not be demonstrated, and these eggs were discarded. Eggs in which only one slit could be found were not recorded as representing zero angle, for although two sperms may conceivably enter by one slit the slit itself cannot be regarded as a potentially reactive zone. Assessments of angles to the nearest 10° were made on a total of 29 eggs and the results were: 20-30°, two eggs; 40-50°, two eggs; 80-90°, four eggs; 100-110°, 10 eggs; 120-130°, seven eggs; 140-150°, three eggs; 160-170°, one egg.

On the assumption that the zona has a spherical surface, that both sperms have a chance of entering by any given area on the surface, and that this chance is proportional only to the area, the probability of both sperms penetrating the same hemisphere is 1/2. This probability will clearly be reduced if the passage of the first sperm induces a reduction in the penetrability of the zona that spreads from the point of entry. Out of 29 eggs, only eight were found with both sperms in the same hemisphere. This is significantly different from equipartition ($\chi^2 = 5{\cdot}83$, $P < 0{\cdot}05$). However, in the fallopian tube the eggs are generally clumped together and sperms would tend first to penetrate the hemisphere of the egg that faces outwards. Therefore the probability of both sperms entering the same hemisphere would, in fact, be greater than 1/2, and the significance of finding only eight eggs penetrated in the same hemisphere would be higher than that just obtained. Thus it can be concluded that the change resulting from sperm penetration is propagated through the zona.

(*c*) *Assessment of the Time Taken for Completion of the Change in the Zona Pellucida of the Rat Egg Consequent upon Sperm Penetration*

To obtain a measure of the rate at which the zona pellucida undergoes change after the entry of the first sperm, mated rats were killed during the period when sperm penetration was taking place and also 24 hr later, when all penetration would have ceased. In each fallopian tube the number of sperms that were present in eggs in excess of one per egg, and the proportion of penetrated eggs, were determined. The zona reaction time was assessed from the mean time between the entry of the first and subsequent sperms into an egg.

Results were obtained from three groups of rats. In the first group, oestrous rats were mated after the time of ovulation; they were placed with males for 30 min between 8.30 a.m. and 9.30 a.m., and those that had copulation plugs were killed 2½, 3, 3½, or 4 hr later. Of the 128 rats killed, 72 yielded penetrated eggs. The data are recorded for the fallopian tubes according to the number of eggs and the number of penetrating sperms in excess of one per egg (Table 3). The mean number of extra penetrating sperms was 0·04 per egg in tubes in which up to 40 per cent. of the eggs were penetrated.

Table 3

NUMBER OF EGGS CONTAINING EXTRA SPERMS, AND TOTAL NUMBER OF EXTRA SPERMS IN THE EGGS’ FROM FALLOPIAN TUBES OF RATS KILLED WHILE PENETRATION WAS IN PROGRESS, OR 24 HR LATER

Type of Mating	Time of Killing	Percentage Penetration per Tube	Number of Tubes	Number of Eggs		Total Number of Extra Sperms	Ratio of Extra Sperms to Penetrated Eggs
				Penetrated	With Extra Sperms		
Delayed	While penetration proceeding	0^{+}-40	41	53	2	2	0·04
		40^{+}-100	74	313	113	167	0·53
	24 Hr after mating	93·1	124	511	159	236	0·46
Normal	While penetration proceeding	0^{+}-40	24	33	2	2	0·06
		40^{+}-100	29	108	19	25	0·23
	24 Hr after mating	98·1	174	808	180	264	0·33

A second group of rats was mated under the same conditions as the first. There were 62 that had copulation plugs and these were killed 24 hr after mating when all sperm penetration would have ceased. The mean number of extra sperms per egg was 0·46 (Table 3).

The rats that made up the third group had been kept under conditions of controlled illumination (Austin and Braden 1954*b*) which was arranged so that ovulation occurred shortly after midday. They were caged with males and mating took place at a normal time in relation to ovulation. Fifty animals were found with copulation plugs and they were killed at 2, 3, 4, or 5 p.m. In the fallopian tubes in which up to 40 per cent. of the eggs were penetrated, the mean number of extra sperms per egg was 0·06 (Table 3). An estimate of the mean number of extra penetrating sperms per egg (0·33) in normally mated rats killed 24 hr after mating is also included in Table 3. This figure is derived from the rats mentioned in Section (*a*) and Table 1.

Upper and lower limits for the rate of change in the zona pellucida were calculated in the following way:

(i) *Lower Limit of Mean Zona Reaction Time.*—A lower limit of T—the time required for the completion of the zona reaction—was derived from the consideration that in fallopian tubes with a low percentage of penetrated eggs at the time of killing (0^+-40 per cent.), a smaller proportion of eggs with extra sperms would be expected than in tubes in which penetration was more advanced, for in the latter the eggs had, in general, been penetrated longer. It was assumed that this difference was due only to the stage at which the animals were killed so that the proportion p' of penetrated eggs that would have had extra sperms could be estimated from data on the rats killed after all penetration had ceased.

In any egg, owing to the change in the zona caused by the first penetration, the probability $p(t)\delta t$ of an extra sperm entering during the time t to $t+\delta t$ from penetration will tend to decrease, reaching virtually zero after time T. Clearly, for a given value of p', a lower limit of T is obtained by taking $p(t)$ constant $(=p)$ for time T and zero thereafter. Then the probability $G(t)$ for an egg penetrated by a single sperm to acquire extra sperms within time t can be shown to be given by

$$\begin{aligned} G(t) &= 1-e^{-pt}, \qquad (0 \leqslant t \leqslant T) \\ &= 1-e^{-pT} \qquad (t > T) \end{aligned}$$

so that p can be estimated from the relation

$$p' = 1-e^{-pT}.*$$

Next, an expression was found for the corresponding probability *at the time of killing*. In any one tube there would generally be one, sometimes two, penetrated eggs, when 0^+-40 per cent. of the eggs are penetrated. These would have been liable to penetration by extra sperms for different unknown lengths of time, t. The distribution $f(t)$ of this 'waiting time' may be taken as approximately exponential, viz.

$$f(t) = ae^{-at} \qquad (0 \leqslant t \leqslant \infty),$$

where $1/a$ is the mean waiting time. The value of $1/a$ can be estimated in the following way. It has been shown that in rats mated after ovulation the mean interval between the penetration of the first and last egg in any one fallopian tube is about 1 hr (Austin and Braden 1954*b*). Since the mean number of eggs per tube was found to be five, the average interval between the penetration of any two eggs would have been about 15 min. A conservative estimate, therefore, of the mean waiting time, $1/a$, would be 5 min. Then the required probability P is

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* This expression for $G(t)$ depends on the assumption, made throughout this section, that sperms penetrate the egg independently.

$$\begin{aligned} P &= \int_0^\infty G(t)f(t)\mathrm{d}t \\ &= \int_0^T (1-\mathrm{e}^{-pt})f(t)\mathrm{d}t+(1-\mathrm{e}^{-pT})\int_T^\infty f(t)\mathrm{d}t \\ &= \frac{p}{p+a}\,(1-\mathrm{e}^{-(p+a)T}). \end{aligned}$$

From the data on the rats mated after ovulation (Table 3) it is seen that in the 0^{+}-40 per cent. class two eggs out of 53 have extra sperms. With $p' = 159/511$ and $1/a = 5$ min, it is easy to calculate for specified values of T the probability P' for the occurrence of extra sperms in two or fewer eggs out of 53. P' is found to be less than 0·05 for $T = 10$ min, which may thus be taken as a lower limit of the mean zona reaction time. Similar calculations for the rats that were kept under conditions of controlled illumination and mated before ovulation show that for $T = 10$ min, P' is less than 0·15. In these rats the mean interval between the penetration of the first and last egg in any one fallopian tube was taken to be 2 hr (Austin and Braden 1954*b*).

(ii) *Upper Limit of Mean Zona Reaction Time.*—A somewhat different approach was used to obtain an upper limit of T. Let the rate of penetration of extra sperms be $r(t)$ per minute per egg at time t from the first penetration. Suppose that $r(t)$ is given by the exponential decay curve

$$r(t) = r_0\mathrm{e}^{-ct} \qquad (0 \leqslant t \leqslant \infty).$$

This allows penetration over an infinite period. T is defined such that all but 5 per cent. of extra sperms will have entered the eggs within time T, so that

$$\int_T^\infty r(t)\mathrm{d}t = 0\cdot05\int_0^\infty r(t)\mathrm{d}t,$$

that is

$$\mathrm{e}^{-cT} = 0\cdot05,$$

or

$$cT = 3\cdot00.$$

c is given by the relation

$$\int_0^\infty r(t)\mathrm{d}t = f,$$

where f is the ratio of extra sperms to penetrated eggs for killings after the cessation of all penetration, an estimate of which is available from Table 3.

Thus

$$r_0/c = f,$$

so that

$$T = 3\cdot 00\, f/r_0.$$

Now it seems reasonable to suppose that the actual decay curve does not decrease more rapidly than the above exponential. An upper limit of T may thus be found from a lower limit of r_0.

As already noted, the mean interval between the penetration of the first and last egg in any one fallopian tube, in rats mated after ovulation, is about 1 hr. During this time the average penetration rate may therefore be taken as at least one sperm per hour per egg. Assuming no sudden change in the zona of an egg at the moment of the penetration of the first sperm, this will give also a lower limit of the initial rate r_0, viz. $r_0 = 1/60$. With $f = 0\cdot 46$ (Table 3) the upper limit of T is found to be about 1½ hr. The result obtained from the rats kept under controlled illumination was similar, namely about 2 hr.

Finally, it may be pointed out that in both groups of rats a lower limit for T of about 10 min is obtained by the present method if $r(t)$ is defined by

$$\begin{aligned} r(t) &= r_0 \qquad (0 \leqslant t \leqslant T) \\ &= 0 \qquad (t > T), \end{aligned}$$

with $r_0 = 1/20$, $1/40$ respectively. As these values correspond to reasonable upper limits of r_0, this result provides a confirmation of the estimate already given.

Table 4

NUMBERS OF SPERMS IN THE EGGS OF RABBITS KILLED 10-20 HR AFTER COITUS

Interval Between Mating and Killing (hr)	Number of Animals	Number of Eggs			Number of Sperms in Perivitelline Space		Number of Sperms in Zona	
		Recovered	Examined	Penetrated	Mean	Range	Mean	Range
10	4	19	9	5	0·2	0-2	0·6	0-2
13	4	30	19	19	12·6	0-29	4·3	0-9
16	6	48	28	28	61·8	10-169	9·3	2-29
20	5	29	18	18	82·5	18-240	9·7	1-29

(*d*) *Number of Penetrating Sperms in the Eggs of the Rabbit, Sheep, and Dog*

Nine eggs were examined from rabbits killed 10 hr after coitus, five were penetrated. The mean number of sperms in the perivitelline space was 0·2 and, in the thickness of the zona, it was 0·6 (Table 4). The mean number of sperms at both sites was much higher in eggs from rabbits killed at 13 hr: 12·6 and 4·3 sperms respectively. The values increased still further in the next 3 hr: 28 eggs examined 16 hr after mating had a mean number of 61·8 sperms in the perivitelline space and 9·3 sperms in the thickness of the zona. The differences between the mean numbers in the perivitelline space at 10 and 13 hr, and also at 13 and 16 hr were significant. There was, however, no significant increase in the mean number in the perivitelline space between 16 and 20 hr.

Twelve eggs were recovered from 16 ewes killed 24-48 hr after service. Of these, six eggs were in various stages of fertilization, three eggs were in the 2-cell stage, and three were in the 4-cell stage. They were observed in the fresh state by microscopy after slight compression under the coverslip. Subsequently they were fixed and stained while still under the coverslip. Several hundred sperms were regularly observed attached to and, in some instances, partially embedded in the zona pellucida, but none was seen in the perivitelline space (Plate 1, Fig. 5).

Twelve eggs in the 6- to 8-cell stage were recovered from the tubes of two bitches 3-4 days after coitus. Eight eggs were examined in the fresh state and four after fixing, sectioning, and staining. As was noted with the sheep eggs, numerous sperms were seen adhering to the external surface of the zona pellucida but none could be detected in the perivitelline space (Plate 1, Figs. 3 and 4).

IV. Discussion

It is rare for more than one sperm to enter the vitellus in the eggs of the marsupial and placental mammals, though under certain experimental conditions the incidence of this phenomenon, polyspermy, may be greatly increased (Austin and Braden 1953*a*, 1953*b*, 1954*a*). The low incidence of polyspermy in normal circumstances has generally been attributed to a propagated change, 'the block to polyspermy,' in the cortex of the egg, which is initiated by the entry of the first sperm and which precludes the entry of subsequent sperms. A similar reaction to sperm penetration is well known in the eggs of invertebrates, where it has been studied in some detail (Rothschild and Swan 1949, 1951, 1952; Runnström and Kriszat 1952). By contrast, the occurrence of a change in the zona pellucida consequent upon sperm penetration does not seem to have been suspected.

In rats and mice, penetration of the zona pellucida by a second sperm occurs much more frequently than penetration of the vitellus by two sperms; in normally mated animals in the present experiments the incidence of polyspermy for both species was 1·2 per cent., whereas in 22·0 per cent. of rat eggs and in 17·4 per cent. of mouse eggs two or more sperms had passed through the zona pellucida. Examination of the distribution of the extra sperms that penetrated the zona showed a departure from what would be expected if chance alone operated; considering the low proportion of eggs with no sperms, the number that had more than one sperm was much smaller than expected. This departure from the theoretical distribution is highly significant and leads to the conclusion that, in rats and mice, the passage of the first sperm through the zona pellucida evokes a reaction which greatly reduces the chances of a subsequent penetration. The reaction could either be a change in the nature of the zona that renders it less penetrable or it could involve a loss of attractiveness of the egg for the sperm. The first alternative seems the more likely because the second implies the existence of chemotaxis between egg and sperm, and there is apparently no sound evidence for this phenomenon in any species of animal (Buller 1903; Tyler 1948; Rothschild 1951). Moreover it is difficult to conceive how a loss of attractiveness could spread in a relatively short time through the large mass of hyaluronic acid in which the eggs are embedded. It is, therefore, highly probable that the zona pellucida of rat and mouse eggs reacts to sperm penetration by becoming less penetrable to further sperms. The evidence obtained also suggests that the reaction is propagated through the zona from the point of entry of the sperm.

Data secured from rats after both normal mating and delayed mating have been used to estimate the time required by the zona reaction to reach completion. From both sets of results the conclusion drawn is that the period is between 10 min and 1½-2 hr. It is considered that the assumptions made in arriving at these figures were particularly conservative and that the mean zona reaction time is very unlikely to lie outside the stipulated limits. It would be of interest to have some idea of the relation of this period to the time required for the change in the vitellus known as 'the block to polyspermy.' Direct evidence is virtually impossible to obtain at present, but indirect evidence suggests that, under normal conditions, the reaction time of the vitellus is shorter than that of the zona. The fact that the incidence of polyspermy in rats and mice is much lower than the incidence of eggs containing more than one sperm is consistent with this suggestion. In the sea-urchin the block to polyspermy is completed in about 1 min (Rothschild and Swan 1952) and it is reasonable to suppose that a similar time may pertain in mammalian eggs. In the rabbit the zona apparently does not react to sperm penetration, yet the incidence of polyspermy after normal mating is no higher than in rats (Austin and Braden 1953*a*, 1953*b*).

As just noted, the zona pellucida of the rabbit egg, in contrast to that of rat and mouse eggs, showed no evidence of a decrease in penetrability for the first 6 hr after ovulation. Indeed the rate of sperm penetration of the rabbit egg increased from six sperms per egg per hour over the first 3 hr to 18 per egg per hour over the second 3 hr. By 6 hr after ovulation a thin layer of mucoprotein ("albumen") begins to accumulate on the eggs (Braden 1952, 1953) (see Plate 1, Fig. 1). This layer presumably prevents the entry of further sperms, as postulated by Hammond (1934), for the mean number of sperms in the eggs at 20 hr was not significantly higher than at 16 hr.

The rabbit appears to be practically unique among mammals in that its eggs commonly have more than 50 sperms in the perivitelline space. The appearance of the rabbit egg is shown in Plate 1, Figures 1 and 2. Information is available for the eggs of 32 species of mammal from nine different orders (Appendix I), and it appears that the eggs of mammals generally display few or no sperms in the perivitelline space, although about 20 sperms have been reported in the mole (Heape 1886) and "several" to "numerous" sperms were noted in eggs of the pocket gopher (Mossman and Hisaw 1940). As was noted in the present investigation, a small proportion of rat and mouse eggs contained up to 10 sperms, and similar observations have been made for the guinea pig (Hensen 1876; Lams 1913), the cat (Hill and Tribe 1924), the ferret (Hamilton 1934), and several species of bats (Van Benedin 1867, 1911; Van Benedin and Julin 1880; Van der Stricht 1910). In all the remaining species, although they were commonly seen in large numbers on the surface of the zona or within its substance, no sperms were found in the perivitelline space. The 12 sheep eggs and the 12 dog eggs examined by the present authors were like this. It appears highly probable therefore that, in most species of mammal, the zona pellucida changes after the entry of the first sperm in such a way as to reduce greatly the chances of penetration by further sperms. However, the rate at which this change occurs in different species evidently varies. Rat eggs, for instance, commonly had sperms in the perivitelline space, whereas sheep eggs had none, and this was so in spite of the fact that the frequency of sperm-egg collisions is of approximately the same order in both the species (Braden and Austin 1954).

As the reaction of the zona to sperm penetration is so widespread among mammals, it is presumably of some significance for the normal development of the fertilized egg. Just as the reaction of the vitelline surface to sperm penetration is important in protecting the egg against polyspermy, so the existence of a similar reaction in the zona pellucida would protect the egg from the entry of excessive numbers of sperms into the perivitelline space. The zona reaction may possibly also provide an additional bar to polyspermy, but only in species in which the reaction occurs most rapidly, for example the dog and the sheep. The effect that large numbers of sperms in the perivitelline space would have upon the egg is not clear, however. Rat eggs with more than 15 sperms were all degenerate in appearance, and Mossman and Hisaw (1940) reported in the pocket gopher an egg containing about 65 sperms that was plainly degenerating. On the other hand, the rabbit egg, and to a less extent, the mole egg, can evidently tolerate the presence of numerous sperms.

In conclusion, it may be pointed out that three mechanisms have now been demonstrated which together ensure that, in mammals, with the exception of the monotremes, the eggs will be fertilized without there being too great a danger of penetration by an excessive number of sperms. Firstly, the chances of meeting of egg and sperm are controlled by the restrictive action of the female tract on sperm passage, so that relatively few sperms are present at the site of fertilization (Austin and Braden 1952; Braden 1953; Braden and Austin 1954). Secondly, the zona pellucida has now been shown to react to the entry of the first sperm so as greatly to reduce the chances of a subsequent penetration. Finally, the entry of the fertilizing sperm into the vitellus evokes a reaction in the cortex that tends to exclude further sperms; evidence for the operation of this 'block to polyspermy' in mammalian eggs has been discussed by Austin and Braden (1953*b*).

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Addendum

After this paper had been submitted for publication, confirmatory evidence of a change in the zona pellucida after sperm penetration was found in a note by Smithberg (1953). In his studies on the effects of proteolytic enzymes on the zona of mouse eggs, Smithberg noted that the zona was always removed more rapidly from unfertilized than from fertilized eggs, and concluded that the nature of the zona pellucida changes after fertilization.

Explanation of Plate 1

Photographs of living eggs, except for that in Figure 4.

Fig. 1.—Rabbit egg examined without compression, showing numerous sperms in the perivitelline space, but none on the surface of the zona pellucida. This egg was recovered about 7 hr after ovulation and already has an appreciable layer of mucoprotein ("albumen") on the surface of the zona. × 400.

Fig. 2.—Rabbit egg compressed beneath coverslip showing four sperms between the vitellus and the zona, and one in the thickness of the zona. × 1000.

Fig. 3.—Dog egg in the 6-cell stage compressed beneath coverslip. Numerous sperms visible on the surface of the zona, but none seen between the vitellus and the zona. × 1000.

Fig. 4.—Section of 8-cell dog egg stained to show sperm heads; these are evident in large numbers on the surface of the zona, but are lacking between the vitellus and the zona. × 400.

Fig. 5.—Sheep egg in the 2-cell stage, slightly compressed. No sperms visible in the perivitelline space, but many are present on the zona. × 1000.

Fig. 6.—Degenerate rat egg containing 50-60 sperms within the bounds of the zona pellucida. Phase-contrast microscopy. × 400.

Appendix I

The following is a summary of information available on the occurrence of extra penetrating sperms in the eggs of mammals. Only reports on eggs that were uncleaved or were in the 2- to 16-cell stages are included.

MARSUPIALIA

Didelphis virginiana.—About 100 eggs examined. Sperms not reported in the perivitelline space (PVS) or the zona, although many were seen in the "albumen" layer (Hartman 1916, 1919). One egg "polyspermic" (Hill 1918).

Setonix brachyurus.—One egg, with no sperms in the PVS but several embedded in the zona; there were many sperms in the "albumen" layer (Sharman, personal communication 1953).

Dasyurus viverrinus.—About 20 eggs examined. No sperms reported in the PVS or the zona, although there were many in the "albumen" layer. One egg was "polyspermic" (Hill 1910).

INSECTIVORA

Talpa europea.—Three eggs illustrated, all shown with about 20 sperms in PVS (Heape 1886).

Blarina brevicauda.—About 40 eggs seen. Sperms often found in the corona but no mention of any in the PVS or zona (Pearson 1944).

CHIROPTERA

Vesperugo noctula.—About 100 eggs. One egg had one sperm in the PVS, the remainder had none (Van der Stricht 1910).

Vespertilio mystacinus.—Three eggs, one of which had 16 sperms in the PVS, the remainder had none (Van Benedin and Julin 1880).

Vespertilio murinus.—Twelve eggs examined. One egg had one sperm in PVS but none embedded in the zona, three eggs had "several" sperms in the PVS, two eggs had a sperm embedded in the zona, but none in the PVS, and the remaining six eggs had no sperms in the PVS or the zona (Van Benedin 1867, 1911; Van Benedin and Julin 1880).

Vespertilio dasycnemus.—Three eggs seen, one had several sperms embedded in the zona, but none in the PVS. The remaining two eggs contained no extra sperms (Van Benedin 1911; Van Benedin and Julin 1880).

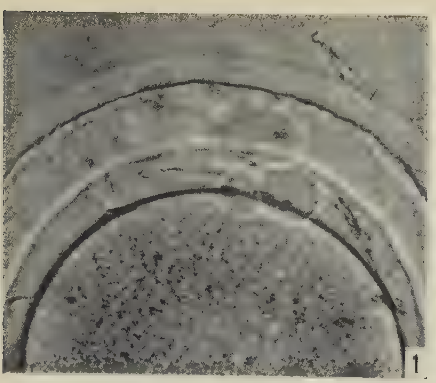
Rhinolophus ferrum-equinum.—Only one egg seen. It had several sperms embedded in the zona, but none in the PVS (Van Benedin and Julin 1880).

PRIMATES

Macaca rhesus.—Four eggs seen. There were apparently no sperms in the PVS, but embedded in the zona were seven, five, and, in two eggs, "several" sperms (Lewis and Hartman 1933, 1941).

Homo sapiens.—Two eggs examined. No sperms reported in the PVS though there were many in and on the zona (Hamilton, Barnes, and Dodds, 1943; Hamilton 1944).

ZONA PELLUCIDA AND SPERM PENETRATION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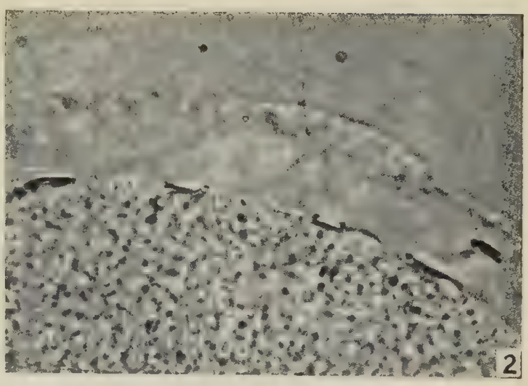
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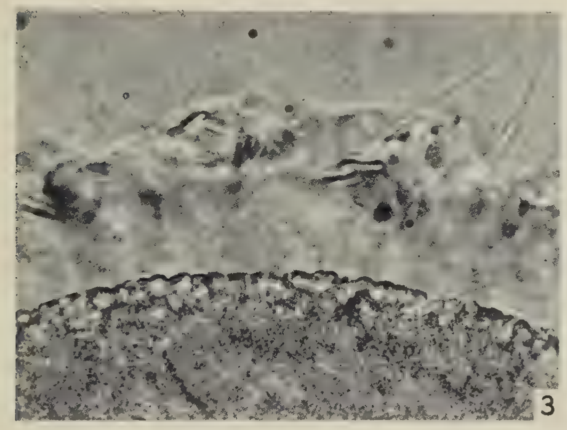
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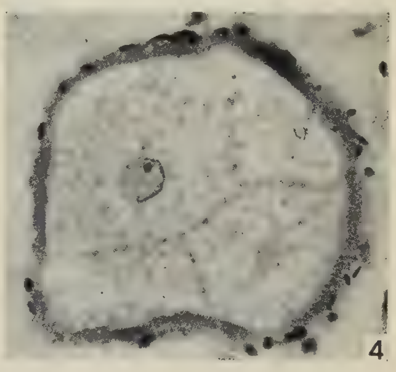
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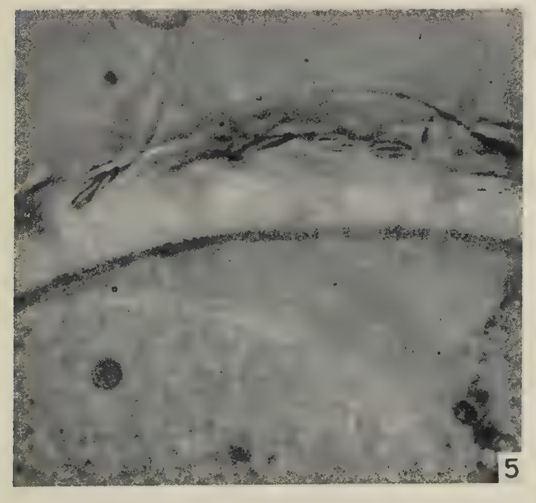
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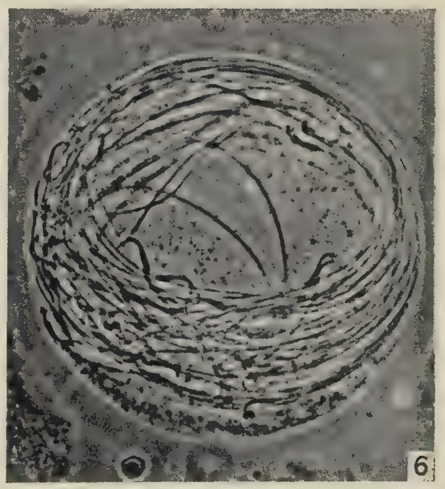
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LAGOMORPHA

Oryctolagus cuniculus.—Eggs from two rabbits examined. In each egg "a number of sperms" were seen in the PVS (Barry 1843).

One egg. In the PVS, 20 sperms were counted in an optical section (Van Benedin 1875).

About 30 eggs seen, and up to 50 sperms were counted in the PVS, and in addition many sperms were seen embedded in the zona (Hensen 1876).

Eight eggs examined. The mean numbers of sperms in the PVS and in the zona 4½-6 hr after ovulation were 37 and 34 respectively (Moricard and Bossu 1949).

About 20 eggs seen. The mean numbers of sperms in the PVS and in the zona 6-14 hr after ovulation were 17 and 10 respectively (Chang 1951).

Forty-six eggs examined. The mean numbers of sperms in the PVS and in the zona 6-10 hr after ovulation were 72 and 9 respectively (Present authors, Table 4).

RODENTIA

Cricetus auratus.—About 40 eggs were examined, but no extra penetrating sperms were seen (Graves 1945).

Geomys bursarius.—Eight eggs examined. There were "several" to "numerous" sperms in the zona and the PVS. One degenerated egg had more than 65 sperms in the PVS (Mossman and Hisaw 1940).

Citellus elegans.—Fourteen eggs seen. There were many sperms embedded in the zona, but none in the PVS (Schooley 1934).

Tamias striatus.—Six eggs seen. Many sperms in the zona, but none in the PVS (Schooley 1934).

Eutamias operarius.—Eight eggs seen. Many sperms in the zona, but none in the PVS (Schooley 1934).

Callospermophilus lateralis.—Eight eggs examined. Many sperms in the zona, but none in the PVS (Schooley 1934).

Mus musculus.—1350 eggs examined. Sperms were rarely seen in the PVS (Sobotta 1895).

Thirty-six eggs examined. Occasional eggs had one or two sperms in the PVS (Lams and Doorme 1908).

Fifteen eggs illustrated. Two eggs can be seen to have two sperms in the PVS, and another two eggs each have one sperm in the PVS (Lewis and Wright 1935).

Eggs examined, 169. Twenty-seven eggs contained one sperm in PVS, one egg had two sperms, one egg had three sperms, and another egg had five sperms in the PVS. Two eggs were dispermic (Present authors, Table 1).

Rattus rattus.—About 150 eggs examined. Sperms were "quite commonly" seen in the PVS (Sobotta and Burckhard 1910).

About 120 eggs seen. "More than one sperm may pass through the zona"; the extra sperms remained in the PVS (Gilchrist and Pincus 1932).

Eggs examined, 366. Fifty-seven eggs contained one sperm in the PVS, 20 eggs had two sperms, five eggs had three sperms, two eggs had four sperms, and one egg had five sperms (Odor and Blandau 1949).

Eggs examined, 810, of which 132 eggs had one sperm in the PVS, 24 eggs had two sperms, 11 eggs had three sperms, three eggs had four sperms, two eggs had five sperms, two eggs had six sperms, and one egg had seven sperms. Two abnormal eggs had 24 and 50-60 sperms respectively in the PVS. Ten eggs were dispermic (Present authors, Table 1).

Cavia porcellus.—About 20 eggs examined. A number of eggs had 1-10 sperms in the zona, but only four eggs had one sperm in the PVS (Hensen 1876).

Fifteen eggs seen. There were no sperms in the PVS (Lams and Doorme 1908).

Eggs seen, 108. Sperms occasionally found embedded in zona and, more rarely, in the PVS. One "polyspermic" egg (Lams 1913).

CARNIVORA

Mustela frenata.—Twenty-five eggs examined. No sperms were reported in the PVS or in the zona (Wright 1948).

Mustela furo.—Five eggs seen. One egg had one sperm in the PVS, another had five sperms, and the rest had none. Sperms were frequently seen in the zona (Hamilton 1934).

Mustela vison.—Four eggs examined. Many sperms were seen on and around the zona, but none reported in the PVS or the zona (Enders 1952).

Felis catus.—Twelve eggs illustrated. None of the eggs shown to have sperms in the PVS, but two had a number of sperms embedded in the zona. In addition, one dispermic egg was described (Van der Stricht 1911).

A small number of eggs was examined. Up to 400 sperms were seen on and around the zona but apparently no sperms were present in the PVS or zona (Longley 1911).

Eleven eggs described. One egg had "several" sperms in the PVS and six eggs had "numerous" sperms in the zona. Two eggs were dispermic (Hill and Tribe 1924).

Canis familiaris.—Seven eggs illustrated and described. Each had numerous sperms on the surface of the zona, but apparently none was seen in the PVS (Bischoff 1845).

Nineteen eggs illustrated. Sperms are shown on the surface of the zona, but not in the PVS (Van der Stricht 1923).

Twelve eggs examined. No sperms were seen in the PVS, although there were many on the surface of the zona (Present authors).

PERISSODACTYLA

Equus caballus.—Five eggs examined. There were a few sperms on the surface of the zona, but apparently none in the PVS (Hamilton and Day 1945).

ARTIODACTYLA

Sus scrofa.—Five eggs seen. Numerous sperms were found adhering to the zona, but none reported in the PVS (Assheton 1899).

Three eggs examined. "Numerous sperm heads in zona." Apparently no sperms in the PVS (Heuser and Streeter 1929).

Twenty-nine eggs examined. Sperms "associated with zona," but apparently no sperms seen in the PVS (Green and Winters 1946).

Bos taurus.—About 30 eggs examined. Many sperms were seen in the zona, but none reported in the PVS (Hamilton and Laing 1946).

Six eggs seen. A "large number" of sperms was reported in and on the zona but none mentioned as being in the PVS (Black *et al.* 1953).

Ovis aries.—Six eggs depicted. Numerous sperms shown on the zona, but none visible in the PVS (Clark 1934).

Twelve eggs examined. There were numerous sperms on the surface of the zona and a few embedded in the zona, but none in the PVS (Present authors).

Capra hircus.—Fourteen eggs described. Numerous sperms were seen on the surface of the zona, but apparently none in the PVS (Amoroso, Griffiths, and Hamilton 1939, 1942).

ADDITIONAL GENES IN *TRITICUM VULGARE* FOR RESISTANCE TO *ERYSIPHE GRAMINIS TRITICI*

By M. V. CARTER*

[*Manuscript received May* 17, 1954]

Summary

Additional genetic studies have been made of the resistance of Birdproof, Chul, and Normandie to races *P* and *P*-1 of *Erysiphe graminis tritici*.

The presence in Birdproof of a second gene, Ml_b, not allelic to the gene Ml_s previously identified in Indian, Sonora, and Sturgeon, has been confirmed. It confers resistance to both races (*P* and *P*-1) of *Erysiphe* from the three-leaf stage onwards.

Chul possesses a single dominant gene, Ml_c, for resistance to race *P*-1 at all stages of growth.

Normandie is shown to possess the Ulka gene, Ml_u, for resistance to both races of mildew.

I. INTRODUCTION

A genetic study of the resistance of 12 varieties of *Triticum vulgare* Vill. to a South Australian isolate of *Erysiphe graminis tritici* has been reported previously by Pugsley and Carter (1953). In this report, the F_2 segregation data from the cross Normandie × Ulka were insufficient to differentiate between a 63 : 1 ratio and a non-segregating population. The cross was therefore repeated and a larger F_2 population secured.

Differential reactions of Chul C.I. 2227 to races *P* and *P*-1 of mildew were also reported: crosses between Chul and Federation and between Chul and representatives of groups *A* and *B* (Pugsley and Carter 1953) were therefore made in order to determine the genetic basis of resistance to race *P*-1 in Chul. Crosses between Chul and group *C* varieties were not made because of the phenotypic difference in the nature of their resistance: Chul is immune at all stages of growth whereas the three varieties comprising group *C* (Indian, Sonora, and Sturgeon) are resistant only after the three-leaf stage.

Additional tests with larger F_2 samples from Birdproof crosses were made in order to clarify previous results indicative of additional resistance in Birdproof.

II. MATERIALS AND METHODS

The varieties used were from the same sources as described by Pugsley and Carter (1953).

Susceptibility tests were carried out as described previously (Pugsley and Carter 1953). All tests involving crosses of which Chul was one parent were made at the Division of Plant Industry, C.S.I.R.O., Canberra, during the winter and spring of 1953 by courtesy of Dr. E. M. Hutton. Race *P*-1 of *E. graminis tritici* had been maintained there on Federation and tests of Chul showed that this race had remained free from contamination by the Chul-attacking race *P*.

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The remaining tests of crosses not involving Chul were made at the Waite Institute, Adelaide, during the winter and spring of 1953 and summer of 1953-54. When daily temperature maxima in the glass-houses became too high for satisfactory mildew infection to take place during the summer, seedlings were inoculated and incubated in the coolhouse where the maximum temperature was kept below 75°F. Race *P* of *E. graminis tritici* was used in all the Adelaide tests.

III. EXPERIMENTAL RESULTS

Results of susceptibility tests of F_2 progeny of the various crosses are given in Table 1.

TABLE 1

CLASSIFICATION OF F_2 SEGREGATES OF THE CROSSES NAMED WITH RESPECT TO MILDEW REACTIONS

Cross	Where Tested	Mildew Race	Resistant	Susceptible	Ratio	Value of *P* for Ratio Indicated
Normandie × Ulka	Adelaide	*P*	338	0	Non-seg.	
Chul × Federation	Canberra	*P*-1	160	40	3:1	>0·10
Thew × Chul	Canberra	*P*-1	212	17	15:1	>0·30
Axminster × Chul	Canberra	*P*-1	216	16	15:1	>0·50
Normandie × Chul	Canberra	*P*-1	344	10	63:1	>0·05
Ulka × Chul	Canberra	*P*-1	210	21	15:1	>0·50
Birdproof × Chul (single-leaf stage)	Canberra	*P*-1	330	16	15:1	>0·20
Birdproof × Chul (3-leaf stage)	Canberra	*P*-1	223	8	63:1	>0·01
Birdproof × Indian (3-leaf stage)	Adelaide	*P*	351	9	63:1	>0·10
Birdproof × Federation (3-leaf stage)	Adelaide	*P*	342*	14	15:1	>0·05

* Of these, 288 seedlings showed no macroscopic symptoms of infection and 54 showed infection of the leaf sheath only (like Sturgeon).

The absence of susceptible segregates in the Normandie × Ulka F_2 sample of 338 seedlings clearly indicates that these varieties share a common gene for mildew resistance. If the second gene in Normandie were different from that in Ulka (Ml_u) the F_2 would show a 63 : 1 segregation. The probability of obtaining no susceptible segregates in an F_2 of 338 seedlings is less than 0·005, so that their absence is highly significant evidence against the second Normandie gene being different from Ml_u.

In the cross Chul × Federation, susceptible segregates constituted approximately one-quarter of the F_2 population, indicating that Chul possesses a single dominant gene for resistance to race *P*-1. F_2 segregations from crosses between Chul and varieties of groups *A* and *B* were all indicative that the Chul gene (designated Ml_c) was not allelic to either of the previously identified genes Ml_t and Ml_u.

Separate tests of F_2 samples of Birdproof × Chul at two stages of growth showed clearly the operation of a second gene for resistance in Birdproof. Although similar in effect, this gene is not allelic with Ml_s (which gives resistance from the three-leaf stage onwards in group *C* varieties Indian, Sonora, and Sturgeon) because susceptible segregates occurred in Birdproof × Indian F_2. The second Birdproof gene is therefore designated Ml_b. Further confirmatory evidence is provided by agreement with a 15 : 1 ratio in the test of Birdproof × Federation inoculated at the three-leaf stage, a previous test made at the single-leaf stage (Pugsley and Carter 1953) having agreed with a 3 : 1 ratio. The presence of leaf-sheath infection (as occurs in Sturgeon) in 54 out of the 342 resistant seedlings in the Birdproof × Federation F_2, suggests the operation of some modifying factor on one of the resistance genes. In the F_2 it is expected that 20 per cent. of the resistant seedlings will carry Ml_t alone, a further 20 per cent. Ml_b alone, and the remaining 60 per cent. both Ml_t and Ml_b. The leaf sheath infection data are most readily explained if it is supposed that there is a dominant gene (*Ls*) also segregating, whose sole effect is to modify the phenotype due to one of the resistance genes so that these seedlings become leaf-sheath-susceptible. If such were the case, three-quarters of 20 per cent., i.e. 15 per cent. of the total resistant seedlings should show leaf-sheath susceptibility. Amongst the 342, the expectation would be 51·3 such seedlings, which is in agreement with the observed number of 54. On present evidence it is not possible to suggest whether *Ls* is carried by Birdproof or Federation.

F_3 families of Birdproof × Federation raised from leaf rust-susceptible mildew-resistant F_2 segregates in the 1952 Canberra tests (i.e. families lacking Ml_t and the associated gene for resistance to race 135AB of *Puccinia triticina*) were found to be of two types. Both were susceptible to race *P* of mildew at the single-leaf stage, but when inoculated at the three-leaf stage it was possible to distinguish families homozygous for Ml_b (all seedlings resistant) from those heterozygous for Ml_b (segregating approximately one-quarter susceptibles).

IV. Discussion

It is noteworthy that Lowther (personal communications, 1951) identified two races of *E. graminis tritici* to which Normandie was resistant but to which both Axminster and Ulka were susceptible. This suggests the presence in Normandie of a third resistance gene distinct from Ml_t and Ml_u. It has not been possible to detect such a gene during the course of the present investigations in the absence of appropriate mildew races.

Summarizing previous results with those reported in this paper, the genotypes of the 13 mildew-resistant varieties may be written as in Table 2.

These five genes for resistance to *E. graminis tritici* may be used as differentials for the separation of mildew races and would permit the identification of $2^5 = 32$ physiologic races.

TABLE 2

GENOTYPES OF WHEAT VARIETIES RESISTANT TO *ERYSIPHE GRAMINIS TRITICI*

Group *A*		Group *C*	
Axminster	Ml$_t$ ml$_u$ ml$_s$ ml$_c$ ml$_b$	Indian	ml$_t$ ml$_u$ Ml$_s$ ml$_c$ ml$_b$
Birdproof	Ml$_t$ ml$_u$ ml$_s$ ml$_c$ Ml$_b$	Sonora	ml$_t$ ml$_u$ Ml$_s$ ml$_c$ ml$_b$
Converse	Ml$_t$ ml$_u$ ml$_s$ ml$_c$ ml$_b$	Sturgeon	ml$_t$ ml$_u$ Ml$_s$ ml$_c$ ml$_b$
Huron	Ml$_t$ ml$_u$ ml$_s$ ml$_c$ ml$_b$		
Kenya C6041	Ml$_t$ ml$_u$ ml$_s$ ml$_c$ ml$_b$	Group *D*	
Thew	Ml$_t$ ml$_u$ ml$_s$ ml$_c$ ml$_b$		
Norka	Ml$_t$ ml$_u$ ml$_s$ ml$_c$ ml$_b$	Chul	ml$_t$ ml$_u$ ml$_s$ Ml$_c$ ml$_b$
Normandie	Ml$_t$ Ml$_u$ ml$_s$ ml$_c$ ml$_b$		
Group *B*			
Normandie	Ml$_t$ Ml$_u$ ml$_s$ ml$_c$ ml$_b$	Federation	ml$_t$ ml$_u$ ml$_s$ ml$_c$ ml$_b$
Ulka	ml$_t$ Ml$_u$ ml$_s$ ml$_c$ ml$_b$		

V. ACKNOWLEDGMENTS

The author wishes to express his thanks to Dr. E. M. Hutton of the Division of Plant Industry, C.S.I.R.O., Canberra, through whose courtesy the 1953 Canberra tests were possible, and to Miss G. M. Roberts, of the Division of Plant Industry, who carried out these tests.

Dr. A. T. Pugsley, of the Agricultural Research Institute, Wagga, kindly read and criticized the manuscript.

The author is grateful to Professor D. G. Catcheside for suggesting a logical explanation of the leaf-sheath susceptibility phenomenon discussed in this paper.

VI. REFERENCE

PUGSLEY, A. T., and CARTER, M. V. (1953).—The resistance of twelve varieties of *Triticum vulgare* to *Erysiphe graminis tritici*. *Aust. J. Biol. Sci.* **6**: 335-46.

CROSS PROTECTION BY STRAINS OF TOMATO SPOTTED WILT VIRUS AND A NEW THEORY TO EXPLAIN IT

By R. J. Best*

[*Manuscript received March* 29, 1954]

Summary

A study of interference between strains of tomato spotted wilt virus in tomato plants has revealed that the mild strain C_1 protects plants against the severe strain A whether this is inoculated before or after systemic symptoms of the mild strain appear (10 and 20 days respectively).

Similarly, the mild strain E protects plants against the severe strain B. However, quantitative determination of yields of fruit and bush has revealed that a significant reduction in yield of both was effected by the severe strain when this was inoculated to plants already infected with the mild strain, although these "protected plants" yielded significantly more fruit and bush than the controls inoculated with the severe strain alone.

When mixtures of strains were inoculated together in the same inoculum the same protective effects were observed and in no case did the severest symptoms appear.

It is pointed out that theories previously put forward to explain the phenomenon of protection do not satisfy the facts described in this paper.

A new theory has been put forward to explain these results and postulates a transfer of character determinants between virus particles at some stage while they are multiplying in the cells they mixedly infect.

I. Introduction

It has been known for some time (Thung 1931; Salaman 1933) that when mild and severe variants or strains of a plant virus exist, infection of a plant with a mild strain may protect it against damage by the severe form. This effect has been used to test suspected relationships between viruses, and it was for this purpose in the first instance that we tested strains of tomato spotted wilt (T.S.W.) virus for their possible cross-protection effects (Best and Gallus 1953). The results of the earlier tests suggested further experiments to test the underlying causes of the effects observed. These are described below along with a new theory to explain the phenomenon of cross protection.

II. Materials and Methods

(*a*) *Strains of Virus*

The strains of T.S.W. virus used in this work were the severe, necrotic, pigmenting types A and B, and the milder, non-pigmenting strains C_1 and E,

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as described by Best and Gallus (1953). These strains had been maintained on suitable host plants in an insect-proof glass-house by serial transfer over a period of years.

(*b*) *Test Plants Used and Growing Conditions*

Lycopersicum esculentum (var. Dwarf Champion) were the tomato plants used unless otherwise stated. They were raised in an insect-proof glass-house capable of being heated in winter. At a suitable stage they were potted up in compost-loam mixture in 6-in. unglazed earthenware pots. They received a weekly addition of ammonium sulphate solution. Plants were normally inoculated at the eight-leaf stage.

Light and temperature in the house vary according to external conditions but the following information will indicate the ranges involved:

Temperature.—The mean daily maximum and minimum temperatures in the glass-house during the experiment did not vary greatly and are set out in Table 1.

TABLE 1

MEAN DAILY MAXIMUM AND MINIMUM TEMPERATURES (°C) IN THE GLASS-HOUSE DURING THE EXPERIMENTS

Month	Quantitative Experiment		Other Experiments	
	Maximum	Minimum	Maximum	Minimum
August	28	12		
September	27	13	30	13
October	27·5	13	25	15·5
November			31	16·5

Light.—The period of bright sunshine during the spring months in the years concerned averaged 5 hr per day.

Plants of *Nicotiana glutinosa* and of *Nicotiana tabacum* were raised under similar conditions but were potted into 4-in. pots. *Glutinosa* plants were normally inoculated from 3 to 5 weeks after potting and tobacco plants from 4 to 6 weeks, according to the time of year.

All seed used was grown and collected in the glass-house in isolated compartments.

(*c*) *Inoculation Procedure*

Tomato plants were inoculated on three leaflets on each of three leaves on the bottom half of the plant. *Glutinosa* plants had the small lower leaves (from 2 to 4) removed, and received inoculum on the next four leaves, leaving the upper portion of the plant untouched. Tobacco plants were inoculated on two smooth lower leaves (usually the first two or the second and third, not counting the cotyledons).

The leaves were normally dusted with celite (abrasive) except for quantitative assays by the primary lesion method. The inoculum was rubbed on lightly with one pass of a ground glass spatula as described in previous papers from these laboratories, and the excess inoculum was washed off immediately with water.

(*d*) *Preparation of Inocula*

The selected plant tissue was removed from the plant, weighed, and either used at once or after cooling it for a few minutes at $-20°C$ in a deep freeze. It was then ground in a mortar in the presence of an equal weight of a buffer solution of about pH 7·5 and 0·01M in respect to a suitable reductant (cysteine or sodium sulphite). The crushed material was transferred to a square of fine book muslin and the juice squeezed through by hand pressure. The volume of squeezate was measured as a check on recovery and additional buffer added to bring the total added to nine times the fresh weight of material. The juice concentration is then approximately 1 in 10.

III. Experimental Results

(*a*) *Severe Strain Applied After the Whole Plant Invaded by Mild Strain*

A batch of tomato plants was inoculated on August 20 with strain C_1 of T.S.W. virus 23 days after the tomato plant had been potted. The first systemic symptoms appeared 16 days after inoculation. All plants showed systemic symptoms 20 days after inoculation and at this stage four plants were inoculated on the lower leaves with strain A of T.S.W. Healthy tomato plants of the same age were inoculated at the same time with an aliquot of the strain A inoculum used above.

After 8 days plants inoculated with A alone developed systemic necrotic, pigmented lesions, stem necrosis, and the other normal symptoms of strain A, leading rapidly to apical necrosis (so-called "tip blight"). One of these plants was used as a source of inoculum for the next experiment to be described. The others died.

The plants which had been invaded by strain C_1 before being inoculated with strain A developed pigmented, necrotic, primary lesions 10 days after being inoculated with A but no symptoms of strain A developed outside the inoculated leaves. These plants grew on, became very bushy, and even 40 days after strain A had been applied showed only the mottle in greens on the top leaves and light ringspots characteristic of the plants infected with C_1 only. The inoculated leaves, however, continued to develop necrotic lesions and by the 29th day most had dehisced. By the 40th day (when observations ceased) all inoculated leaves had fallen off.

It is clear that plants infected with the mild strain were protected against the severe strain.

(*b*) *Severe Strain Applied Before Systemic Symptoms of Mild Strain Appeared*

In the previous experiment the plants had been infected with the mild strain 20 days before the severe one was applied. The next experiment was designed to test the effect of applying the severe strain *before* systemic symptoms of the mild one were visible, so an interval of 10 days between inoculation with the mild and severe strains was chosen. Ten tomato plants were drawn from each of two batches, one of which was 7 days younger than the other. On September 9 six plants of each batch were inoculated with strain C_1 of T.S.W. virus. Ten days later eight of these plants were inoculated with the severe strain A, four plants being drawn from each batch. At the same time another eight plants, four from each batch, which had so far not received any treatment were inoculated with strain A only. The remaining four plants of the original 20 were kept as controls.

Those plants which had received strain C_1 first and then strain A developed symptoms typical of strain C_1 in due course, there was a shortening of the internodes and flattening of the leaves and leaflets, accompanied by the appearance of non-pigmented ring-spots and an etch pattern on the surface of the sub-upper leaves, together with a chlorotic mottle on the upper leaves. These plants developed brown necrotic primary lesions on the inoculated leaflets but no symptoms of strain A showed at any time on the rest of the plants. The inoculated leaves gradually fell off. The plants grew on to the flowering and fruiting stages although all were shorter and less bushy than the healthy, untreated controls.

The plants that had received strain A only developed systemic symptoms typical of that strain, culminating in apical necrosis: 30 days after inoculation with strain A they were dead, whereas at this time the plants that had received strain C_1 and then strain A were all well covered with leaf and were fruiting.

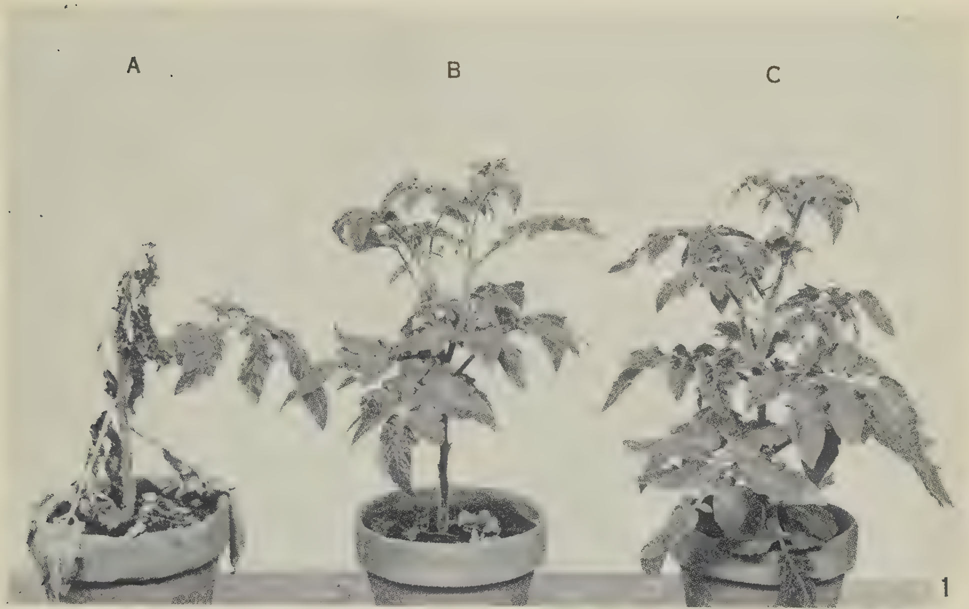
Plate 1, Figure 1, shows the protective effect clearly.

One interesting observation was made which was sufficiently striking to be recorded although its significance was not recognized at the time. Tomato plants when grown and inoculated under our conditions normally develop visible systemic leaf symptoms only in the upper half of the plant except at a very late stage. On this occasion some beautifully developed white and green concentric rings developed on a lower leaf of one plant at a time when normally no symptoms would be expected on this part of the plant. The leaf had not been inoculated. The plant concerned had received strain A after being infected with strain C_1. The ringspot symptoms are shown in Plate 1, Figure 2. The plants inoculated with strain C_1 alone did not develop this type of symptom.

(*c*) *Quantitative Measure of the Effects of Protective Infection*

The protective effect of the mild strain on a subsequent inoculation with a severe one was so marked and obvious that no quantitative estimations were made, it being considered more important at that stage to carry the observations on as long as possible. However, because the protected plants seemed often to be less leafy than their controls a further experiment was carried out so that any effects could be measured quantitatively.

CROSS PROTECTION BY STRAINS OF T.S.W. VIRUS



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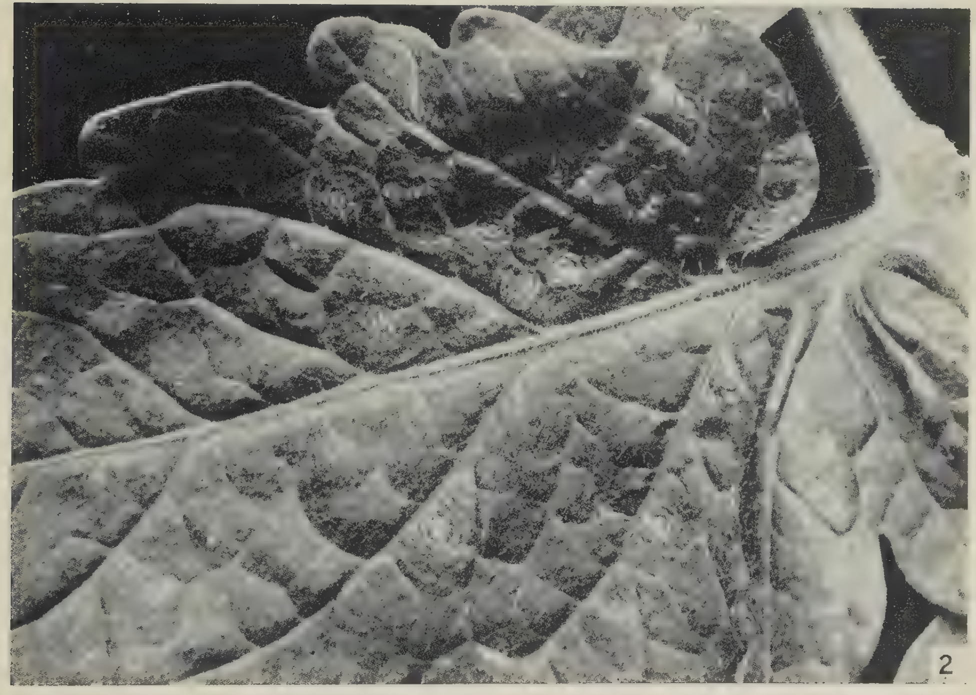

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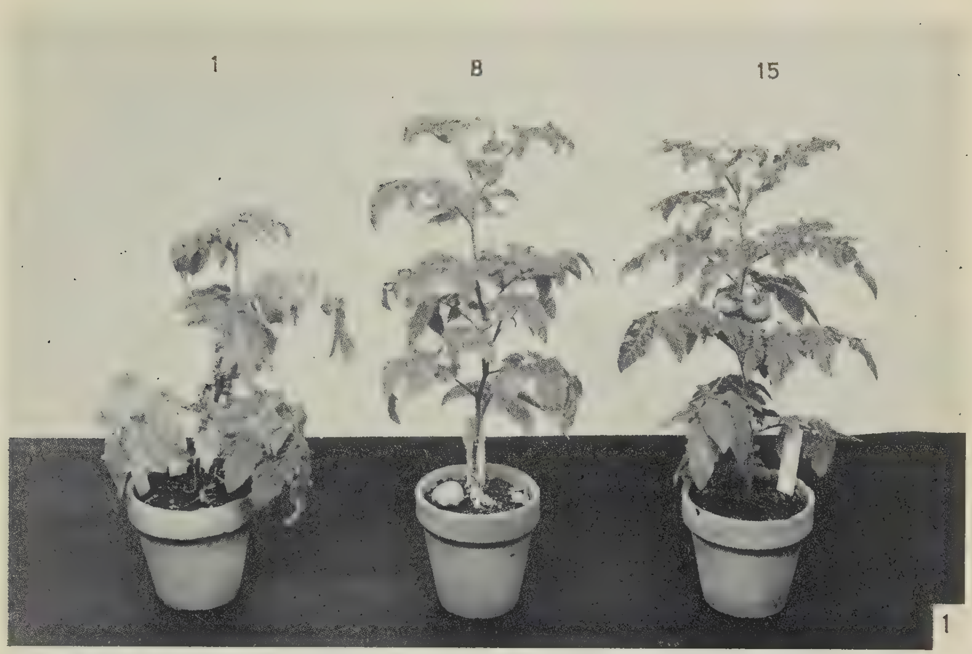
CROSS PROTECTION BY STRAINS OF T.S.W. VIRUS



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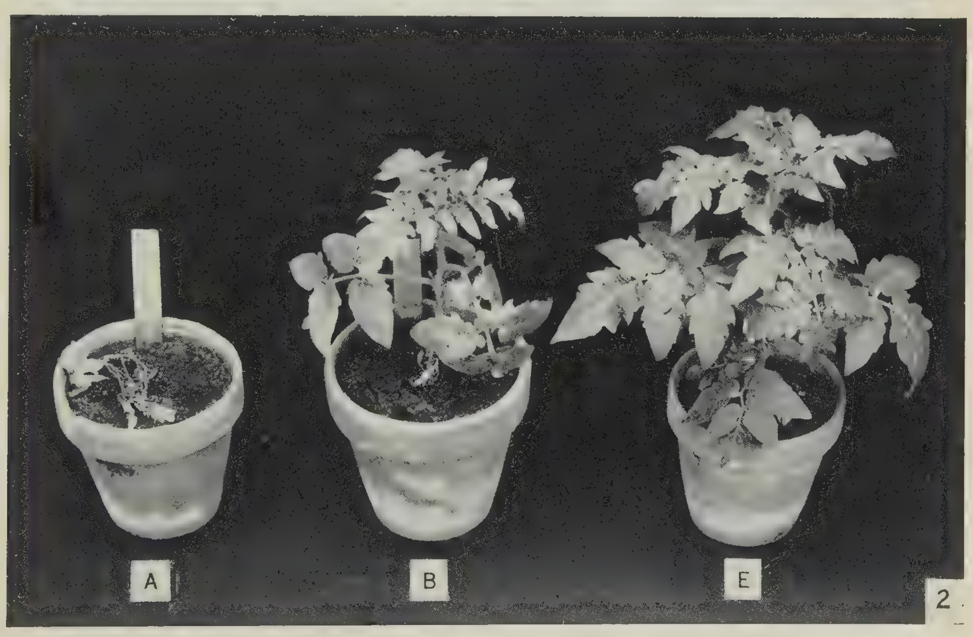

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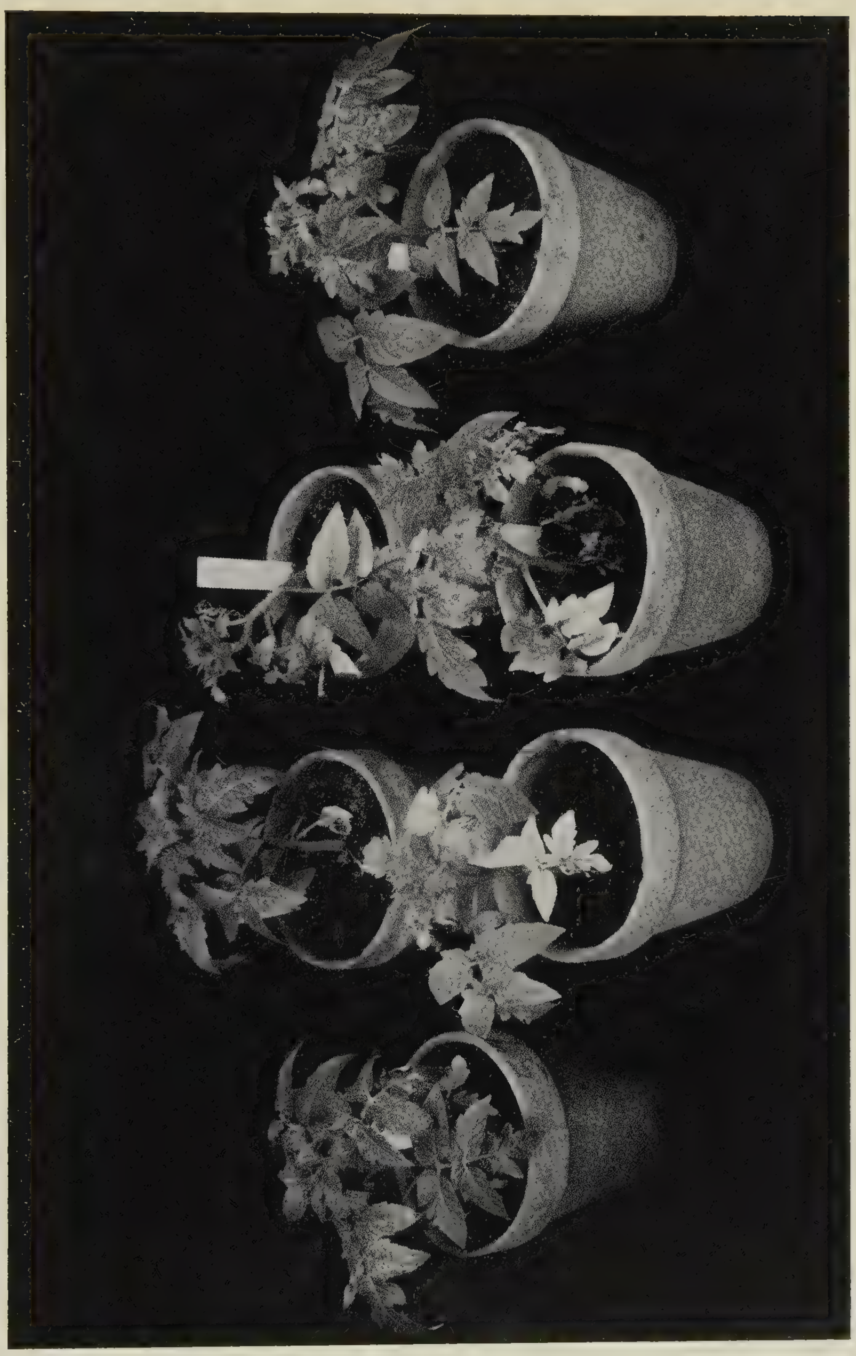
PLATE 3

CROSS PROTECTION BY STRAINS OF T.S.W. VIRUS



Twenty tomato plants at the eight-leaf stage were arranged into four batches of five plants each. One batch was kept as healthy controls, the second batch received an inoculum of T.S.W. strain E, the third batch was inoculated with strain E at the same time as batch 2 and with strain B 19 days later, the fourth batch was inoculated with strain B only at the same time as this strain was applied to batch 3.

The plants inoculated with single strains developed symptoms typical of the strain used. The plants inoculated with both strains developed symptoms of the milder one only, with the exception that one leaflet on one plant of batch 3 curled downward and developed brown necrotic lesions. The plants within each batch were reasonably uniform in appearance, and representative samples of these were photographed as shown in Plate 2, Figure 1, 68 days after the first inoculation.

Seventy-five days after the beginning of the experiment (i.e. after the first inoculation) the plants were harvested and fresh and dry weights of the fruit (still green) and above-ground portions of the plants were determined. The results are set out in Table 2.

TABLE 2

INFLUENCE ON YIELD OF TOMATO PLANTS OF INFECTING THEM WITH A MILD AND A SEVERE STRAIN OF TOMATO SPOTTED WILT VIRUS ALONE AND TOGETHER

Each number represents the weight (g) of fruit or above-ground portions of five plants

Treatment	Fresh Weight			Dry Weight		
	Fruit	Leaves and Stem	Total	Fruit	Leaves and Stem	Total
Healthy controls	302	492	794	31·5	65·9	97·3
Strain E	212	393	605	19·5	46·1	65·6
Strain E, then strain B	122	313	435	11·2	40·6	51·8
Strain B	38	203	241	4·4	31·1	35·5

It should be noted that the protective effect of strain E on strain B is statistically significant. Nevertheless, plants inoculated with the severe strain B after strain E has spread into them systemically were markedly affected by B. The fruit yield is almost halved and total fresh weight is reduced by about a third.

N. glutinosa plants arranged in four batches of four each were treated similarly to tomato plants described above but strain B was inoculated 12 days after E. Of the *N. glutinosa* plants inoculated, those which received B only reacted with the usual pigmented necrotic primary lesions, and the virus did not go systemic in any plant. Similarly, plants inoculated with E only reacted with non-necrotic, chlorotic blotches on the inoculated leaves some time later, and developed systemic chlorosis. Those plants to which strain B was applied after strain E developed systemic symptoms more nearly comparable with plants which had received E only, but the symptoms were more severe and one plant developed some systemic necrosis.

(*d*) *Effect of Infecting Tomato Plants Simultaneously with Strains A, B, and E*

It is of considerable practical importance as well as academic interest to know how soon the protective effect of a mild strain can work. So an experiment was carried out in which the strains whose interactions were being studied were applied at the same time.

A batch of 32 tomato plants at the eight-leaf stage was divided into eight batches of four. The plants were then inoculated according to the following plan:

Batch No. - -	1	2	3	4	5	6	7	8
T.S.W. strains in inoculum - -	A	B	E	A+B+E	A+B	A+E	B+E	Control

Plants which received one strain only developed symptoms appropriate to that strain, whereas the mixedly infected plants developed symptoms which did not conform to the pattern of any of the single strains but resembled those of a mild strain B more closely than any other. In no case did a mixedly infected plant develop apical necrosis or die, even when strain A was a constituent of the infecting inoculum. Apart from those which received strain A alone, only one plant developed pigmented stem necrosis. This plant had received a mixture of A and E, which mixture appeared to be slightly more severe than any of the other mixtures.

The effects are illustrated in Plate 2, Figure 2, and Plate 3.

The inocula used above were also applied to plants of tobacco and *N. glutinosa* and the primary lesions produced on these were counted. The results are recorded in Table 3.

IV. DISCUSSION

In the experiments just described four strains of T.S.W. virus were concerned, the pigmenting necrosing strains A and B and the milder ring-spotting strain C_1 and chlorosing strain E. The mild strains protect plants against the severe ones when the latter are inoculated before or after the former have spread systemically throughout the plant, and even when inoculated simultaneously with them.

One of the earliest accounts of a protective effect of one strain of a plant virus on another is that of Thung (1931), who observed a protective effect between two strains of tobacco mosaic virus when one was inoculated before the other, and claimed that the second one was completely excluded. Salaman (1933) observed a similar effect between two strains of potato virus X, and suggested the following explanation of the phenomenon: "the secondarily inoculated and severer type has failed to gain an entry. It would appear that once the plant cell has formed a symbiotic union with the non-virulent form, it has no capacity to enter into relations with any other virus elements of the same generic type." Valeau (1941), working with tobacco ring-spot virus, concluded that the so-called "acquired immunity" effects could be satisfactorily explained on the basis of competition. The general idea of competition seems to be the most popular of the theories advanced to explain the interference phenomenon. The first strain in uses up the raw materials, essential building blocks, and the like, so there is nothing left for the second one to multiply on. Plausible as this might appear nobody has produced any experimental evidence to support it.

Table 3

PRIMARY LESIONS PRODUCED ON PLANTS OF *LYCOPERSICUM ESCULENTUM* (VAR. DWARF CHAMPION), *NICOTIANA TABACUM* (VAR. BLUE PRYOR), AND *NICOTIANA GLUTINOSA*

These were inoculated with single strains of tomato spotted wilt virus, and with mixtures of strains applied in the one inoculum. When more than one strain was involved the inoculum was prepared by mixing together equal volumes of each

Strain	Primary Lesions per Leaf (average of six)			
	Tomato Necrotic	Tobacco Necrotic	*N. glutinosa*	
			Necrotic	Yellow Blotch
A	7	(Leaves collapsed >40)	23	0
B	0	48	18	0
E	0	24	0	2·1
A+B+E	0	70	11	0·8
A+B	0·9	11	25	0
A+E	1·8	17	12	1
B+E	0·3	21	18	0·5

The quantitative experiment described in this paper (Table 2) proves conclusively that the second strain (B) had a profound effect on the yield of fruit and bush after the first strain (E) had become thoroughly established throughout the plant. It could scarcely have done this if it had not itself invaded the plant systemically and multiplied sufficiently to reduce growth and fruiting significantly, as it did. The theory that the first strain to gain entry to a cell monopolizes some essential building block is, therefore, disproved by this experiment, as also are similar theories based on the same general idea.

Bawden and Kassanis (1945), working with severe and mild etch viruses and the viruses of potato Y and hyocyamus 3, found cross-protective effects in varying degrees, and produced evidence to show that the secondarily inoculated strain can enter and multiply in the plant. To explain their results they advanced the theory that "different viruses combine with and multiply at different specific sites whereas related strains combine with the same sites. If a site in a susceptible cell is already occupied by one strain then a second strain of that virus will be unable to attach itself and multiply." This neat explanation seems more reasonable than others advanced but even it does not satisfy all our own observations.

Another possible explanation of cross protection, namely, that the presence of the first strain so alters the metabolism of the host plant that the second one to be applied cannot evoke its normal symptoms, can scarcely apply when both strains are inoculated together and the fast-moving strain as well as the slow one has its symptoms altered. This explanation will not fit the experimental facts presented in this paper.

In the quantitative experiment described earlier in the paper the normal symptoms of the second strain (B) were not evident when applied after the mild strain E but was masked. It has become fashionable to dismiss many such observations and phenomena by using the word "masked" as if this explained the facts. Actually it explains nothing. The word 'masked' is a convenient and accurate general description of a phenomenon, but nothing more, we still need to find an explanation for the masking. In a previous paper the author (Best 1954) has pointed out that the characteristic symptoms of a particular virus are due to character determinants on the virus. In the experiment referred to above the masking of the pigmentation and the stem and leaf necrosis normal to strain B on tomato plants can be understood if the determinants for these characters are exchanged some time during the developmental or multiplication processes for some of the milder determinants of the mild strain *E*. A strict exchange would not be necessary so long as there is a transfer of determinants from one particle to another. In the above example strain E particles are present in excess of B so it is reasonable to expect the resultants or 'progeny' of interaction to be mainly mild in nature. The fact that the yield from plants inoculated with B after E is depressed below that from those inoculated with E only leads to the conclusion that the new strains formed by the interchange of character determinants are intermediate in severity between strains B and E.

The results of experiments with strains A and C_1 can be interpreted similarly.

These conclusions are supported by the results of the experiment dealing with the effects of mixedly infected plants when two or three strains were inoculated simultaneously onto the test plants. When inoculated alone the severe strain A moves much faster than the mild strain E. The conclusion that the severe strain A of T.S.W. virus moves faster in the plant than the mild strain E is based on tests for the presence of infective virus in various parts of the plant at suitable times after inoculation as well as on appearance of symptoms, it being realized that the latter criterion alone is not adequate. It is reasonable to assume that if the two strains multiplied independently in the inoculated plant the quick-moving severe strain would go systemic first and apical necrosis and death would develop before the slow-moving strain E could get to the top. In practice this has not yet happened. As shown by this experiment the severest effects of strain A (apical necrosis) did not show up in any of the plants inoculated with any mixture of strains whether they contained strain A or not.

The virus remains in the inoculated leaf several days before it moves into other parts of the plant and becomes systemic. We conclude then that while they are developing and multiplying in the inoculated leaf something happens to the strains to change their ability to evoke their characteristic symptoms in other parts of the plant. The results shown in Plate 2, Figure 2, and Plate 3 and accompanying text are readily understood if this something is a transfer of character determinants between the virus particles. It should be noted in Plate 3 that the batch inoculated with a mixture of strains A and E is represented by two plants. This is because all but one were very like the plant in the front row and devoid of stem necrosis but one plant, the one in the back row, did develop pigmented stem necrosis. It was the only plant in the whole experiment which did so after receiving a mixed inoculum. These facts are in agreement with the theory here put forward and suggest that some characters (such as apical necrosis) may be the result of more than one determinant acting together on the one virus particle.

One prediction from the theory is that from plants infected with two strains of the virus we should be able to recover strains which differ from the original infecting strains. This we have done (Best and Gallus, unpublished results). From mixedly infected tomato, *N. glutinosa,* and tobacco plants we have separated strains which, while differing from both "parents," combine some of the characters of each. The fact that these new strains "breed true" on further serial transfer eliminates a possible alternate explanation that the difference in symptoms between 'protected' or mixedly infected plants and those infected by the single strains only is entirely due to a changed reaction of the host without any change in the virus particle. This work is still in progress and will be reported later.

It should be noted that "synergistic action" between virus strains, as noted by many workers in the literature and more recently by Norris (1951) and Finlay (1952, 1953) for T.S.W., can be explained by the theory now being advanced, and would, in fact, be expected if it were true. We have also obtained evidence in support of this in our experiments with mixedly infected plants. There may of course, be other forms of synergism, resulting from the operation of other mechanisms. A full description and masterly discussion of cross protection, so-called "acquired immunity," and the interference phenomenon generally is contained in Bawden's book (Bawden 1950).

Bawden (1934) has shown that a severe strain of potato virus X can be introduced into rapidly growing plants even when these already contain a mild strain. This has been confirmed by Bald (1948). Both authors explain this by suggesting that the severe strain invaded cells or tissues not already invaded by the mild strain. These observations have no bearing on my theory, which is concerned with the interaction of strains which invade the *same* tissues, but they serve very well to illustrate an interesting point noted in the literature describing the symptoms of plants infected with two strains of a virus, namely, that there is evidence in these descriptions that there has been some reduction in severity of the severe strain even when it is re-isolated. This would be expected as normal by the theory advanced in this paper.

V. ACKNOWLEDGMENTS

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EXPLANATION OF PLATES 1-3

PLATE 1

Fig. 1.—Showing the protective effect of strain C_1 of tomato spotted wilt virus on plants of *Lycopersicum esculentum* (var. Dwarf Champion) when subsequently inoculated with the severe strain A of the virus. Plants *b* and *c* were inoculated with strain C_1 of T.S.W. virus on September 9; *a* and *b* were inoculated with strain A 10 days later. The plants were photographed 45 days after the first inoculation. The pots have a diameter of 6 in. at the top.

Fig. 2.—Systemic green and white ring-spot symptoms on a lower leaf of a tomato plant inoculated 45 days earlier on leaves below this one with strain C_1 of tomato spotted wilt virus and 10 days later with strain A of the same virus. The leaf shown did not receive any inoculum.

PLATE 2

Fig. 1.—Effect of previous infection of tomato plants with a mild strain E of tomato spotted wilt virus on subsequent inoculation with a severe strain B of the virus. Plants 8 and 15 were inoculated with strain E of T.S.W. virus on September 12 and plants 1 and 8 were inoculated with strain B 19 days later. The plants were photographed 68 days after the first inoculation.

Fig. 2.—Effect on plants of *Lycopersicum esculentum* (var. Dwarf Champion) of infecting them with single strains of tomato spotted wilt virus. From left to right: strain A, strain B, strain E. All plants, in 6-in. pots, were photographed 25 days after inoculation.

PLATE 3

Effect on plants of *Lycopersicum esculentum* (var. Dwarf Champion) of infecting them with mixtures of strains of tomato spotted wilt virus applied together in the one inoculum. Front row (left to right): A + B + E; A + B; A + E; B + E. The two plants in the back row were from the batches inoculated with A + B and A + E and are included to show the range of variation of symptoms within these batches. All plants in the other batches were very uniform in appearance. The plants were grown in pots with a top diameter of 6 in. and were photographed 25 days after inoculation.

THE ROLE OF MOLYBDENUM IN NITRATE REDUCTION IN HIGHER PLANTS

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Summary

Experiments are described which were designed to locate the molybdenum-requiring step in the chain of reactions involved in nitrate utilization in higher plants.

Tomato plants deficient in molybdenum were treated with molybdate, and the changes in content of nitrate, nitrite, ammonia, amides, amino acids, and proteins, expressed on a dry weight basis, were followed over a short period. Two days after treatment with molybdate there was a marked decrease in the level of nitrate. During this period the concentrations of nitrite, ammonia, amides, amino acids, and protein increased. The nitrite and ammonia contents of the tops were doubled 4 hr after supplying molybdate to the molybdenum-deficient plants.

It is concluded that in the intact plant molybdenum is essential for a reaction involved in the reduction of nitrate to nitrite. The known metabolic functions of molybdenum are discussed briefly.

Analytical methods used in these determinations are described.

I. INTRODUCTION

Although it has been known for some time that molybdenum is essential for the utilization of nitrate by plants (Steinberg 1937; Hewitt and Jones 1947; Mulder 1948) no satisfactory evidence has been presented to indicate the precise step in this process for which molybdenum is required.

Hewitt, Jones, and Williams (1949) showed that not only protein but also amino acid content in molybdenum-deficient plants was low in comparison with that of normal plants, and concluded that molybdenum was required for some step between the initial reduction of nitrate and the formation of amino acids. However, since the comparisons were made between healthy and deficient plants, the possibility that the differences observed were due to the different physiological age of the plants could not be excluded. Mulder (1948) avoided this complication by comparing deficient plants with those which had been treated with molybdate only 3 days prior to harvest, and produced evidence which confirmed the conclusions of Hewitt, Jones, and Williams (1949).

Since in molybdenum-deficient plants nitrate accumulates and protein level is reduced (Mulder 1948), it follows that molybdenum is required directly or indirectly in at least one of the reactions in the sequence of reactions through

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which nitrate is reduced and protein is formed. The experiments reported here were designed to investigate further the role of molybdenum in utilization of nitrate by studying, at short time intervals, the changes in content of possible products of nitrate utilization (viz. nitrate, nitrite, ammonia, amides, amino acids, and proteins) in plants recovering from molybdenum deficiency.

II. METHODS

(*a*) *Water Culture Methods*

Seeds of tomato (*Lycopersicon esculentum* Mill. var. Pan America) were germinated on waxed mosquito netting over distilled water. After approximately three weeks, seedlings were transplanted into nutrient solutions in 3-l. Pyrex beakers. Eight plants were grown per beaker in an unheated glass-house.

The composition of the nutrient solutions and methods of purification of salts used were the same as described earlier (Spencer 1954).

Two weeks after transplanting, i.e. 5 wk from commencement of germination, plants showed typical early symptoms of molybdenum deficiency (Stout and Meagher 1948). No tissue damage was apparent in the plants used for the experiments. Molybdenum-treated controls and molybdenum-deficient plants showed little difference in size at this stage, but the leaves of the former were a healthy dark green colour. Plants were kept in a glass-house throughout the experimental period.

(*b*) *Experimental Design*

General procedure and design varied only slightly in the three experiments reported here.

Experiment 1.—A 3 × 2 factorial design of the following treatments:

Harvests: at zero time (9 a.m.) and at 48 and 96 hr after applying molybdate treatment.

Sodium molybdate: nil, or 0·0151 g $Na_2MoO_4.2H_2O$ per beaker ($\equiv$ 2 p.p.m. Mo) added at zero time.

At each harvest the stems of the plants were cut just below the cotyledons, and tops and roots collected separately. At each harvest the material from seven replicate beakers was bulked to make one sample from each molybdate treatment; the fresh weight was recorded and the material dried for 1 hr at 80°C under a forced draught. Dry weights were determined and the material was then ground finely, by hand, prior to analysis for protein N, amino acid N, amide N, ammonia N, and nitrate N. Roots were analysed for protein N and non-protein N (total N minus protein N) only.

Experiment 2.—A 7 × 2 factorial design of the following treatments:

Harvests: at zero time (7 a.m.) and 2, 4, 8, 12, 24, 36, and 48 hr after applying molybdate treatment.

Sodium molybdate: nil, or 0·0151 g $Na_2MoO_4.2H_2O$ per beaker ($\equiv$ 2 p.p.m. Mo) added at zero time.

The experimental material consisted of 40 beakers divided into two duplicate blocks of 20, each beaker containing eight plants. Within each block 10 beakers were treated with molybdate at zero time and 10 were left untreated. At each harvest one plant was taken from each beaker; this gave 10 plants each from molybdenum-treated beakers and from untreated beakers. Within each group of 10 plants, four plants were used for dry weight determination, and two lots of three plants were used for duplicate ammonia and amide determinations. Plants were severed immediately above the cotyledons, and only tops were analysed. Dry weight was determined after drying at 90°C for 3 hr under a forced draught.

Experiment 3.—An 8 × 2 factorial design of the following treatments:

Harvests: at zero time (9 a.m.) and 1, 2, 3, 4, 5, 6·5, and 8 hr after applying molybdate treatment.

Sodium molybdate: nil, or 0·0151 g $Na_2MoO_4.2H_2O$ per beaker (≡ 2 p.p.m. Mo) added at zero time.

The experimental material consisted of two beakers each containing eight plants. At zero time one beaker was treated with sodium molybdate and the other left untreated. At each harvest one plant was removed from each beaker, its fresh weight recorded, and nitrite assay carried out immediately. Since it was not possible to handle a large number of replicates on the one day, the experiment was repeated on four successive days. The results given are for one day's experiment only, but the same pattern of concentration change was obtained in each of the four replicate experiments.

(*c*) *Analytical Methods*

(i) *Nitrate N.*—This was determined colorimetrically by a modification of the phenoldisulphonic acid method of Burrell and Phillips (1925). An aqueous extraction of the dried plant material was substituted for extraction with 80 per cent. ethanol since smaller amounts of plant pigments were co-extracted by this method. Removal of chlorides with excess silver sulphate was omitted since the experimental material was grown in water cultures to which only minute amounts of chloride had been added and no interference due to chlorides was detected in preliminary investigations.

(ii) *Protein N.*—Dried material (0·5-1·0 g) was extracted with boiling water at pH 4·5 as described by Wood and Sibly (1952). The aqueous extract containing non-protein N was made up to 50 ml. Protein N in the residue was determined by Kjeldahl digestion, using selenium as a catalyst, and subsequent estimation of ammonia in a Parnas-Wagner steam distillation unit, using 2 per cent. boric acid solution to trap the ammonia (Ma and Zuazaga 1942).

(iii) *Amino Acid N.*—This was determined by a combination of the methods of Schlenker (1943) and Sobel, Hirschman, and Besman (1945). To a 5-ml aliquot of the aqueous extract (*supra*) of the dried plant material were added 1 ml H_3PO_4 (2 parts 78-80 per cent. H_3PO_4 + 1 part H_2O), and 0·5 ml (≡ 30 mg) ninhydrin. These were heated in a boiling water-bath for 1 hr in a tube fitted with a capillary stopper. The contents of the tube were next cooled, 3 drops of octyl alcohol added, and the tube inserted in an aeration train (Sobel, Mayer, and Gottfried 1944). Finally 6 ml saturated KOH were added, and air was drawn through the system for 2 hr. The liberated ammonia was trapped in either 1·5 ml 2 per cent. boric acid, and measured by titration, or in 1·5 ml N/50 H_2SO_4 and nesslerized. The free ammonia, and the amide N which is liberated in this procedure, were corrected for by blank determinations on aliquots of plant extract in absence of ninhydrin. A blank must also be run on each batch of ninhydrin. This method detects none of the α-amino N of asparagine and only 10 per cent. of that of glutamine.

(iv) *Amide N.*—This was determined by the method of Schlenker (1943). One ml of 6N H_2SO_4 was added to a 5-ml aliquot of the aqueous non-protein extract, the test-tube stoppered with a capillary stopper, and heated for 3 hr in a boiling water-bath. The digestion tube was connected with an aeration train (Sobel, Mayer, and Gottfried 1944), ammonia was liberated with 5 ml 40 per cent. KOH, and determined as described in the previous section. This result must be corrected for free ammonia, which is determined separately. The value so obtained refers to amide-group N and was doubled to include that of the α-amino group of the amino acid amides. In Experiment 2, the residue from the ammonia assay was used to determine amide N.

(v) *Ammonia N.*—In Experiment 1 this was determined on a 10-ml aliquot of the aqueous non-protein extract using the aeration train mentioned above (Sobel, Mayer, and Gottfried 1944). The aliquot was neutralized to approximately pH 7, and 1·1 ml of a soda-borate mixture (5 g $Na_2B_4O_7$ in 100 ml N/2 NaOH) was added. The tube was connected with the aeration train which was placed in a water-bath at 40°C, and air drawn through the system for 4 hr. Ammonia was trapped in 1·5 ml of 2 per cent. boric acid, and measured by back titration with N/50 HCl. Using pure solutions of ammonium sulphate and glutamine under these conditions it was found that glutamine interfered slightly and gave high results. Fifty μg NH_3 N plus 50 μg glutamine amide N gave 102 per cent. recovery of NH_3 N while ammonium solutions alone gave 93 per cent. recovery.

For Experiment 2 a more reliable method for the determination of ammonia N was developed. The analyses were carried out on fresh plant material, and no interference from glutamine was detectable. From 1 to 2 g fresh leaf were ground in a mortar and transferred to a test-tube with successive 2, 1, 1, 1 ml washings of ethanol. Contents of the tube were heated in a boiling water-bath for 3 min. The alcohol prevents enzymic formation of ammonia in the extracts, but interferes in the subsequent nesslerization if not removed. Phosphate buffer (0·5 ml) (0·3M Na_2HPO_4 adjusted to pH 8·8-9·0) was added to the test-tube, followed by 0·2 ml 1N NaOH and 2 drops of antifoam (1 g "Span 20" + 30 ml liquid paraffin (Archibald 1944)). The tube was connected with an apparatus for reduced pressure distillation, and the ammonia distilled off into 2 ml N/50 H_2SO_4. An essential feature of the reduced pressure distillation apparatus is the inclusion of a water-cooled reflux condenser approx. 10 in. high between still and receiver tubes. Distillation was carried on for 20 min with the still tube immersed in a water-bath at 40°C, and the pressure held at 40 mm, a continuous air stream being drawn through the system. The resulting ammonium solution was made up to 10 ml in a graduated flask, and 0·5 ml Nessler's reagent (Vanselow 1940) added. After 4 min the percentage transmittance of the solution was measured in a Coleman Spectrophotometer Model 14 at 450 mμ.

(vi) *Nitrite*.—The stem of a single plant was severed above the cotyledons, the epicotyl was weighed (0·2-0 7 g) and immediately ground in 1 ml 1N NaOH + 0·5 ml H_2O in a small mortar. The entire grindate was transferred quantitatively to a centrifuge tube with 2·5 ml water given in successive washings of 0·5, 0·5, 0·5, and 1·0 ml. Lead acetate (1·0 ml 1N) was added and, after mixing well, the resulting precipitate was centrifuged down at low speed and the clear, colourless supernatant was decanted into a stoppered measuring cylinder. The residue was resuspended twice in 2-ml aliquots of water, and recentrifuged, the supernatant being decanted into the stoppered cylinder each time. Perchloric acid (2·0 ml 1N) was then added to adjust the pH between 1·0 and 2·0, and the total volume adjusted with water to a convenient amount (usually 12·0 ml). The nitrite present in the extract was determined by a modification of the method of Bratton and Marshall (1939) for the determination of sulphanilamide. To a 5-ml aliquot of the extract was added 1 ml M/250 sulphanilamide and after standing 10 min at room temperature in diffuse light, 1 ml M/250 N-(l-naphthyl)-ethylene diamine dihydrochloride. The transmittance of the resulting pink solution was read at 550 mμ in a Coleman Universal Spectrophotometer. A further 5-ml aliquot of the plant extract was treated similarly but without the sulphanilamide. This served to correct for absorption at 550 mμ due to substances other than nitrite.

In recovery trials in which nitrite was added to the ground plant material it was found that the above lead hydroxide precipitation procedure yielded consistently 60-70 per cent. of the added nitrite, and experimental values obtained were therefore corrected by a factor for 65 per cent. recovery. Other methods of extraction, e.g. trichloracetic acid precipitation, gave even lower recoveries owing to adsorption of nitrite on the precipitate.

(*d*) *Expression of Results*

The analytical data for all metabolites estimated were placed on a dry weight basis (mg N per g dry weight) except those for nitrite N, which were expressed on a fresh weight basis (μg NO_2^- per g fresh weight). The term "content" applied to any metabolite in this paper indicates its amount expressed on these bases.

III. RESULTS

The results of Experiment 1 (Fig. 1) are in agreement with the known effects of molybdenum on nitrate utilization, and provide further evidence for the site of action of molybdenum in this process. The dry matter of roots and tops was increased in the presence of added molybdate. Nitrate accumulated to high concentrations in molybdenum-deficient plants, and protein level in these plants, which was low at zero time, decreased further during the experimental period. The addition of molybdenum resulted in a rapid decrease in the level of nitrate in the tops of the deficient plants, and an accompanying increase in the protein content. Forty-eight hr after the addition of molybdate the nitrate content was reduced to approximately one-quarter of that in the untreated molybdenum-deficient tops. In the same period there was a marked increase in the content of amino acids, amides, and to a less extent of ammonia. Between the second and fourth days after the addition of molybdate, while the nitrate content continued to decrease, and that of protein to increase, the content of the three earlier products of nitrate utilization dropped markedly.

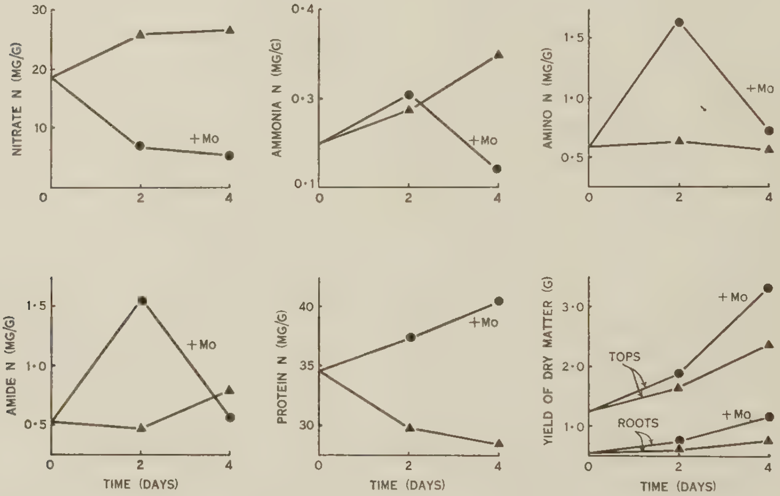

Fig. 1.—Effect of sodium molybdate on the content of nitrate N, ammonia N, amino N, amide N, and protein N in the tops of molybdenum-deficient tomato plants (mg N per g dry weight), and on the yield of dry matter. Sodium molybdate added to one set of deficient plants at zero time. Experiment 1.

Although the increase in ammonia content in the molybdenum-treated plants was less marked than that of the other products, the similarity in trend suggested that the ammonia concentration had risen at a stage earlier than the 48-hr harvest. However, there was a possibility of interference in the ammonia estimation from amides which were present in approximately 10 times the concentration of ammonia. In order to clarify these points, a more satisfactory method of ammonia estimation was developed (*vide supra*), and Experiment 2 was carried out to follow the changes in amounts of the ammonia and amide fractions of molybdenum-deficient plants at short time intervals after treatment with molybdate. The results (Fig. 2) showed unequivocally that there was a rapid increase in ammonia content in the tops of molybdate-treated plants whilst content in the untreated plants remained constant. The concentration of ammonia is roughly doubled 4 hr after addition of molybdate to the water cultures, and reached a maximum value after 8 hr. The amide content increased less rapidly than that of ammonia.

Since the results of Experiment 2 suggested that the molybdenum-requiring reaction occurred prior to the formation of ammonia, it was of interest to study the change in nitrite content under similar conditions. The results of Experiment 3 (Fig. 3) showed that, following the addition of molybdate to molybdenum-deficient plants, the nitrite content of the tops increased rapidly, while the amount of nitrite in the untreated plants was relatively unchanged. In the treated plants the nitrite content rose to a maximum 3-6 hr after the addition of molybdate, and fell to its previous level after 8-12 hr.

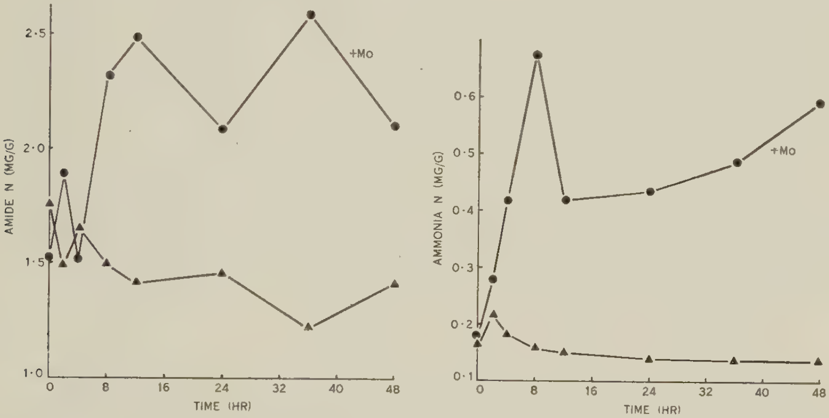

Fig. 2.—Effect of sodium molybdate on the content of ammonia N and amide N in the tops of molybdenum-deficient tomato plants (mg N per g dry weight). Sodium molybdate added to one set of deficient plants at zero time. Experiment 2. The differences in ammonia levels due to molybdate treatment are significant at $P < 0{\cdot}001$ from 4 hr onwards. The differences in amide levels are significant at $P < 0{\cdot}01$ from 8 hr onwards.

It should be noted that the time scales of the separate experiments are not strictly comparable, since experiments were not carried out under controlled environmental conditions.

IV. Discussion

Although the precise chain of reactions involved in the reduction of nitrate and its subsequent incorporation into protein is not fully known, one possible sequence for the formation of the main products of nitrate utilization is

$$\begin{tikzcd}
\text{nitrate} \arrow[r] & \text{nitrite} \arrow[r] & \text{ammonia} \arrow[r] & \text{amino acids} \arrow[r] \arrow[d, shift left] & \text{proteins.} \\
 & & & \text{amides} \arrow[u, shift left] &
\end{tikzcd}$$

It is known that molybdenum is required for some reaction in the above sequence since molybdenum-deficient plants accumulate large quantities of nitrate and are deficient in protein (Mulder 1948). It could be expected that in a molybdenum-deficient plant all products in the above sequence prior to the molybdenum-requiring step would accumulate, and that on adding molybdate to such a plant the concentration of these products would decrease, while that of all products subsequent to the molybdenum-requiring reaction would increase at least temporarily. The present experiments, therefore, studied the changes in concentration of these compounds in molybdenum-deficient plants at short time intervals following treatment with molybdate.

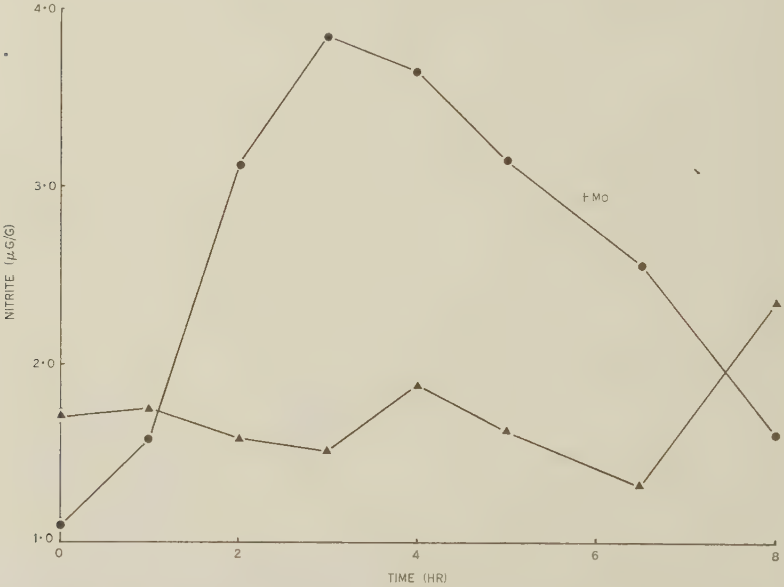

Fig. 3.—Effect of sodium molybdate on the content of nitrite in the tops of molybdenum-deficient tomato plants (μg NO_2 per g fresh weight). Sodium molybdate added to one set of deficient plants at zero time. Experiment 3.

The immediate response to molybdate added to a molybdenum-deficient plant was a decrease in the content of nitrate, and an increase in the contents of nitrite, ammonia, amino acids, amides, and proteins. It follows that the reaction which is retarded in the absence of molybdenum is involved directly or indirectly in the initial reduction of nitrate to nitrite. Thus in a molybdenum-deficient plant nitrate accumulates, since the initial reduction of nitrate becomes the rate-limiting step. Upon addition of molybdenum to the plant the rate of this reaction is increased, and the amount of nitrate decreased, while that of protein and earlier products of nitrate utilization increases. The subsequent decreases in the content of nitrate, ammonia, amino acids, and amides is presumably due to adjustment to new steady state conditions with increased rate of protein synthesis, and an increased supply of carbon acceptor compounds for the product of nitrate reduction. A similar temporary accumulation of an intermediary nitrogen metabolite was reported by Hamner (1935). On treating nitrogen-deficient tomatoes with nitrate Hamner observed a rapid rise in the nitrite content after 6-36 hr, followed by an equally rapid decrease.

The position of ammonia in the above sequence of reactions is not definitely established, and an alternative mode of formation from side reactions, such as the oxidative deamination of glutamic acid, would be equally consistent with the increased ammonia content observed in the present experiments. This alternative would not affect the above interpretation of the role of molybdenum in nitrate utilization, since it is probable that ammonia is formed later than nitrite in the sequence of reactions.

Since the present experiments were carried out, workers at the McCollum-Pratt Institute have isolated from *Aspergillus* and *Neurospora* a nitrate reductase, a flavoprotein enzyme which, in the cell-free, purified state, catalyses the reduction of nitrate to nitrite in the presence of a source of reduced coenzyme (Evans and Nason 1952). Furthermore, it has been shown that molybdenum is an integral part of this enzyme system, the enzyme being inactivated by dialysis against cyanide, and reactivated by the addition of sodium molybdate or molybdenum trioxide (Nicholas, Nason, and McElroy 1953). An enzyme with similar catalytic properties has been isolated from higher plants (Evans and Nason 1953). It is also of interest that recently it has been shown (Richert and Westerfield 1953) that animal xanthine oxidase, an enzyme which is capable of reducing nitrate to nitrite *in vitro,* in a molybdenum-containing flavoprotein.

The role of molybdenum in plant metabolism is now becoming clearer. It appears that molybdenum functions in at least three metabolic systems. It is known to be required for the nitrogen-fixation reactions in both free-living and symbiotic nitrogen-fixing bacteria (Bortels 1930; Jensen and Spencer 1947; Anderson and Thomas 1946). The work of Nicholas, Nason, and McElroy (1953), together with the present experiments, indicates a second function of molybdenum as a component of the nitrate reductase system of plants. Since non-leguminous plants utilizing forms of nitrogen other than nitrate still have a positive molybdenum requirement (Agarwala 1952), at least one other function for molybdenum in plant metabolism must exist. A third function was suggested by Spencer (1954) who showed that, both *in vitro* and *in vivo,* molybdate, in physiological concentrations, inhibits the acid phosphatases of higher plants. It is possible that molybdate thus exerts a sparing action on key phosphorylated metabolites, such as the glycolytic intermediates and adenosine triphosphate, all of which were shown to be hydrolysed by plant acid phosphatases *in vitro.* However, until the action of acid phosphatases within the plant cell is better understood, the effect of their partial inhibition by molybdate remains speculative.

V. ACKNOWLEDGMENTS

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WATER-SOLUBLE CONSTITUENTS OF FRUIT

I. SOME OBSERVATIONS ON THE OCCURRENCE OF FREE GALACTURONIC ACID IN FRUIT

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Summary

Glucuronic acid showed no tendency to convert to the lactone when run on paper chromatograms in a variety of solvent mixtures. There were only slight differences between the R_F values of glucuronic and galacturonic acids in all the solvents used, including two mixtures for which large differences in R_F had been recorded.

By the use of paper chromatography, a free uronic acid was detected in several varieties of pears after ripening at 20°C, but not in green or tree-ripened pears. Similar results were obtained with freestone peaches. No free uronic acid was detected in apricots whether ripened on the tree or at 20°C.

Chemical tests applied to the free uronic acid in pears, after elution from paper chromatograms, indicated that it was galacturonic acid. In the sample which gave the strongest uronic acid spot, the concentration of free galacturonic acid was 350 μg/g fresh fruit.

I. Introduction

Derivatives of polygalacturonic acid are important cell-wall constituents of fruit but, until recently, there has been no evidence for the occurrence of free galacturonic acid among the soluble constituents of fruit.

Harris (1948) isolated galacturonic acid from rotten apples as the *p*-bromphenylhydrazine salt of its *p*-bromphenylhydrazone and, using a colorimetric method, found 1580 μg/ml of free uronic acid in juice from rotten apples and 13-54 μg/ml in juice from different varieties of sound apples. Harris suggested that the estimation of free uronic acid could be used to establish whether rotten apples had been used in the manufacture of juices and jellies. This work was continued by Winkler (1949, 1951, 1952) and Mills (1951, 1952) who found, for example, 1149 μg/g of free uronic acid in rotten pears and 40-52 μg/g in fresh strawberry juice. These authors used colorimetric methods to estimate the uronic acid in the total acids separated as the lead salts, or by using ion-exchange resins; consequently they did not obtain direct evidence that the free uronic acid in sound fruit was galacturonic acid.

This paper presents same evidence that the free uronic acid in sound ripe fruit is galacturonic acid and that its occurrence may depend on the method of ripening. The investigation is being continued with the aim of isolating the free uronic acid. The effect of different methods of ripening is outside the scope of the work and is not being further investigated.

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II. METHODS

(*a*) *Fruit Extracts*

Samples of 6-20 (usually 12-20) sound fruits were used for the preparation of 80 per cent. alcoholic extracts. Cut tissue was dropped straight into a Waring Blendor containing alcohol cooled to -20°C. The mixture was blended for 4 min, bringing the temperature to about 20°C, and then filtered or centrifuged. This procedure was used to minimize enzyme action in the fruit and chemical reactions in the extract. Judging only by paper chromatograms, the same results were obtained with ethanol at room temperature, or when the blended mixture was boiled for 10 min with, or without, calcium carbonate. The extracts were stored at -20°C and gave apparently identical chromatograms over long periods of storage.

(*b*) *Paper Chromatography*

Spots were put on filter paper sheets (24 by 24 in.) with calibrated haemocytometer pipettes or an "Agla" microsyringe. Chromatograms were developed by descending flow in apparatus similar to that described by Woiwod (1949). The tanks were housed in an inside room where the variation in temperature was generally less than 1°C during each run; the annual temperature range was 17-28°C. The solvent was allowed to drip off the bottom edge of the paper (Jermyn and Isherwood 1949) which was serrated with pinking scissors. The papers were dried in a fume-cupboard at room temperature.

The solvents used in all experiments, except those described in Section III (*a*), were ethyl acetate-pyridine-water (2·5 : 1 : 3·5 v/v) (McFarren, Brand, and Rutkowski 1951), *n*-butanol-acetic acid-water (4 : 1 : 1 v/v), and phenol-water 4 : 1 w/v).

The spots were revealed by means of the following reagents:

(i) *Silver Nitrate-Sodium Hydroxide.*—The reagent of Trevelyan, Proctor, and Harrison (1950) was used and the papers were then fixed with sodium thiosulphate (10 per cent.) containing sodium metabisulphite (1·5 per cent.). To obtain maximum sensitivity it was necessary to use a freshly prepared solution of alcoholic sodium hydroxide for spraying.

(ii) *Aniline Phosphate.*—Orthophosphoric acid (3 ml of 98 per cent.) was added to a solution of aniline (6 ml) in water-saturated butanol (600 ml) (cf. Bryson and Mitchell 1951). The sprayed chromatogram was heated for 5 min at 105-110°C. Uronic acids gave pinkish orange spots which appeared as dark spots in ultraviolet light.

(iii) *Naphthoresorcinol-Trichloracetic Acid.*—One volume of naphthoresorcinol (1 per cent. in ethanol) was mixed with 9 volumes of trichloracetic acid (10 per cent.) in water immediately before use. The sprayed chromatograms were heated for 8 min at 95-100°C. The mixture used by Partridge (1948) was less sensitive.

(*c*) *Elution of Compounds from Paper Chromatograms*

The spots of the required compound were located in the usual way (cf. Dent 1947, p. 244) by spraying marker strips, three strips being adequate for a 24-in. chromatogram. The apparatus used for elution was similar to that described by Dent (1947), except that a short piece of filter paper was held between two microscope slides to act as a feeder strip. The strip to be eluted was touched against the wet end of the feeder strip and thus hung from it. Strips were sprayed to test whether the compound had been completely eluted; collection of 1 ml of eluate was found adequate. With one feeder strip, the collection of 1 ml took ½-2 hr, depending on the length of the strip being washed. The rate of elution was increased when desired by using two or three feeder strips superimposed.

III. Results

(*a*) *Paper Chromatography of Galacturonic and Glucuronic Acids*

(i) *Preparation of Glucuronic Acid Solution.*—A solution of sodium glucuronate was prepared by the electrometric titration of glucuronolactone to pH 8 with sodium hydroxide (Heald 1952; Hirsch 1952). The salt was converted to the free acid by shaking the solution with a cation-exchanger ("Zeokarb 225"). This solution of glucuronic acid contained no lactone (i.e. it gave no lactone spot on chromatograms run in butanol-acetic acid-water) immediately after preparation, and was completely stable at room temperature for at least several days.

(ii) *Formation of Glucuronolactone.*—Aqueous solutions (1 per cent.) of glucuronic and galacturonic acids were refluxed for 30 min and then compared on paper chromatograms run for 64 hr in butanol-acetic acid-water. Glucuronic acid was partially converted to the lactone (cf. Quick 1927; Hirsch 1952) as shown by the appearance on the chromatogram of a second spot which ran the same distance as authentic glucuronolactone. This spot was well separated from that due to glucuronic acid. Galacturonic acid was unchanged.

(iii) *Attempted Separation of the Uronic Acids on Paper.*—Glucuronic and galacturonic acids were not separated by butanol-acetic acid-water (4 : 1 : 1 v/v) or water-saturated *iso*butyric acid (cf. Partridge 1948), ethyl acetate-pyridine-water (2·5 : 1 : 3·5 v/v) (cf. Isherwood and Jermyn 1951), ethyl acetate-acetic acid-water (3 : 1 : 3 v/v) (cf. Rao, Beri, and Rao 1951), acetic acid-water (2·5 : 1 v/v), or ethanol-*iso*butyric acid-water (4 : 1 : 1 v/v). When run in amyl alcohol-pyridine-water (7 : 7 : 6 v/v) (Werner and Odin 1949; Masamune and Maki 1951) on acid-washed No. 1 Whatman paper at 22°C, there was a slight difference in the distance run but the separation was incomplete (e.g. distance from starting line after a 48-hr run, glucuronic acid 11·3, galacturonic 9·8 cm; after a 64-hr run, glucuronic 25·0, galacturonic 23·4 cm).

Both acids gave normal spots when run in water-saturated *iso*butyric acid or acetic acid-water on unwashed No. 1 or No. 4 Whatman paper; in all of the other solvent mixtures used, both acids gave elongated or double spots on unwashed No. 1 or No. 4 Whatman paper.

TABLE 1

OCCURRENCE OF A URONIC ACID IN FRUIT (DETECTED ON PAPER CHROMATOGRAMS RUN IN ETHYL ACETATE-PYRIDINE-WATER)

Sample*	Fruit	Variety	Season	Maturity†		Ripened at 20°C (days)	Uronic Acid‡
				Picked	Extracted		
1*a*	Pears	Winter Cole	1950	Green	Green	—	—
1*b*	Pears	Winter Cole	1950	Green	Ripe	9	++
2	Pears	Winter Cole	1950	Green	Ripe	10	++++
3*a*	Pears	Winter Cole	1951	Green	Green	—	—
3*b*	Pears	Winter Cole	1951	Green	Ripe	10	+
4*a*	Pears	Winter Cole	1951	Green	Green	—	—
4*b*	Pears	Winter Cole	1951	Green	Ripe	13	+++
5*a*	Pears	Winter Cole	1952	Green	Green	—	—
5*b*	Pears	Winter Cole	1952	Ripe	Ripe	—	—
5*c*	Pears	Winter Cole	1952	Ripe	Overripe	—	—
5*d*	Pears	Winter Cole	1952	Medium	Ripe	4	+
5*e*	Pears	Winter Cole	1952	Green	Ripe	9	++
6*a*	Pears	Packham's Triumph	1950	Green	Green	—	—
6*b*	Pears	Packham's Triumph	1950	Green	Ripe	10	++
7*a*	Pears	Packham's Triumph	1952	Green	Green	—	—
7*b*	Pears	Packham's Triumph	1952	Medium	Ripe	8	+
7*c*	Pears	Packham's Triumph	1952	Green	Ripe	13	+++
8*a*	Pears	Josephine	1950	Green	Green	—	—
8*b*	Pears	Josephine	1950	Green	Ripe	10	+
9	Peaches	Blackburn Elberta	1950	Ripe	Ripe	—	—
10	Peaches	Blackburn Elberta	1951	Green	Ripe	6-8	+++
11	Peaches	Blackburn Elberta	1952	Medium	Ripe	*c.* 4	+
12	Peaches	J. H. Hale	1950	Green	Ripe	*c.* 8	+
13	Peaches	Golden Queen	1950	Medium	Ripe	*c.* 4	—
14	Apricots	Trevatt	1950	Medium	Medium	1	—
15	Apricots	Trevatt	1951	Green	Ripe	4-5	—
16*a*	Apricots	Trevatt	1952	Green	Green	—	—
16*b*	Apricots	Trevatt	1952	Ripe	Ripe	—	—
16*c*	Apricots	Trevatt	1952	Green	Ripe	5	—
16*d*	Apricots	Trevatt	1952	Medium	Ripe	4	—
17*a*	Apples	Granny Smith	1951	Green	Green	—	—
17*b*	Apples	Granny Smith	1951	Green	Ripe	35	—

* Samples of fruit received are indicated by numbers. Letters indicate subsamples obtained by sorting or ripening the fruit received.

† "Green" fruit had been picked at commercial maturity but was not suitable for eating raw. "Ripe" fruit was eating-ripe. Sample 5 (200 lb of fruit) was cold-stored at −1°C for 4 months and when opened was sorted into green, medium, ripe, and overripe; total solids contents of samples extracted were 5*a*, 15·3; 5*b*, 20·8; 5*c*, 20·1; 5*d*, 18·4; 5*e*, 16·6; confirming that there had been a wide range of maturity at picking. Sample 7 was stored at 0°C and then sorted into green and medium; total solids contents of samples extracted were 7*a*, 17·2; 7*b*, 18·2; 7*c*, 17·5 per cent.

‡ The approximate range of uronic acid contents was 0·4-2 μg in the spot on the paper, corresponding with approximately 60-300 μg/g fresh fruit. The relative values were assessed visually on papers sprayed with silver nitrate-sodium hydroxide.

(*b*) *Detection of a Uronic Acid in Fruit Extracts*

Crude 80 per cent. alcoholic extracts consistently gave compact spots on paper chromatograms. The uronic acid was recognized by its R_F values and its reactions with the three reagents described (Section II (*b*)). Other ions reacted only with the silver nitrate reagent and did not interfere with the recognition of the uronic acid spots (cf. Plate 1). The spots considered to be the uronic acid were not affected in any way by the removal of cations with a cation-exchange resin ("Zeokarb 225" or "Dowex 50"), but disappeared after the removal of anions with an anion-exchange resin ("Amberlite 1R-4B"). The results obtained with the various extracts are set out below:

(i) *Two-dimensional Chromatograms.*—Alcoholic extracts (approx. 60 μl, equivalent to 12·4 mg fruit) of ripe apricots, peaches, pears (3 lots), and apples were applied to No. 1 Whatman filter paper. The chromatograms were run for 48 hr in phenol-water and then 64 hr in butanol-acetic acid-water and were compared with chromatograms of known sugars run under the same conditions. The peaches and two lots of pears had a uronic acid spot corresponding in position to galacturonic or glucuronic acid. There was no spot in the area where glucuronolactone would lie.

(ii) *Chromatograms Run in Phenol-Water.*—Alcoholic extracts (approx. 30 μl, equivalent to 6·2 mg fruit) of green pears, ripe pears, ripe peaches, ripe apricots, green apples, and ripe apples (8, 7, 4, 3, 2, and 1 samples respectively) were applied to No. 1 Whatman paper. The chromatograms were run for 48 hr. Uronic acid spots, corresponding with galacturonic or glucuronic acid, were detected in ripe pears and ripe peaches.

(iii) *Chromatograms Run in Ethyl Acetate-Pyridine-Water.*—As before, approximately 30 μl of each extract was applied to No. 1 Whatman paper. The chromatograms were run for 16 or 24 hr. The results obtained are shown in Table 1 and, for samples 3, 4, and 7, in Plate 1, Figures 1 and 2. Known amounts of galacturonic acid were added to a pear extract which apparently contained no uronic acid, and the chromatograms were sprayed with silver nitrate-sodium hydroxide; 0·2 μg was barely detectable, 0·4-2 μg covered the range given by the fruit extracts.

(*c*) *Tests to Determine the Identity of the Uronic Acid in Pears*

(i) *Collection of Uronic Acid.*—The 80 per cent. alcoholic extract of sample 2 (Table 1) was concentrated under reduced pressure. Spots of the concentrate (equivalent to 31 mg fresh fruit) were applied to No. 3 MM Whatman paper. Chromatograms were run in ethyl acetate-pyridine-water and marker strips were sprayed. The uronic acid was eluted from appropriate strips cut from the unsprayed sections of the papers.

(ii) *Attempt to Form a Lactone.*—Aqueous solutions (1 per cent.) of this uronic acid which had been refluxed for 30 min did not give a lactone spot on paper chromatograms (cf. Section III (*a*) (ii)).

(iii) *Ehrlich Basic Lead Acetate Test.*—This test (Ehrlich 1932) was applied to eluted uronic acid (600 μg) dissolved in water (0·05 ml). When a freshly prepared saturated solution of basic lead acetate (2 drops) was added a white precipitate formed which became orange after heating at 100°C. The solution evaporated, leaving an orange residue. A similar quantity of authentic galacturonic acid gave the same colour and glucuronic acid gave a pale yellow. An eluate from a blank paper run in ethyl acetate-pyridine-water gave no colour.

(iv) *Dische Cysteine Colour Reaction.*—Authentic galacturonic acid gave the characteristic green colour described by Dische (1948), but the same sample, run on paper in ethyl acetate-pyridine-water and then eluted, failed to give the green colour. The reaction also failed when an eluate from a blank paper, which had been irrigated with ethyl acetate-pyridine-water, was added to a solution of galacturonic acid.

(*d*) *Quantitative Determination of Galacturonic Acid in Pears*

Nineteen spots (each 15 μl = 3·1 mg fruit) of an alcoholic extract of sample 2 (Table 1) were put on a sheet of No. 1 Whatman paper. The chromatogram was run in ethyl acetate-pyridine-water, three marker strips were sprayed, and duplicate strips, each containing eight uronic acid spots, were eluted. The eluates were evaporated and the galacturonic acid estimated by the Dische carbazole reaction, using the proportions of sulphuric acid and water given in the modified method (Dische 1950) but heating at 100°C for 20 min (Dische 1947). The sulphuric acid used was heated strongly with ammonium sulphate to eliminate oxides of nitrogen which gave high blank values. Very good agreement was obtained between the duplicates, the average being 320 μg/g. The estimation was repeated using a concentrated extract of sample 2, each spot being the equivalent of 15·5 mg fruit. The mean of good duplicates was 380 μg/g.

IV. Discussion

(*a*) *Paper Chromatography of Glucuronic and Galacturonic Acids*

Using an aqueous solution of glucuronic acid containing no trace of the lactone, it was found that the acid was stable at room temperature for at least several days, and was only partially converted to glucuronolactone by refluxing for 30 min (Section III (*a*) (i) and (ii)) (cf. Quick 1927; Hirsch 1952). Glucuronic acid was also stable when run on paper chromatograms in a variety of solvent mixtures, as shown by the absence of streaking and of the lactone spot.

Partridge (1948) first described the behaviour of glucuronic acid and its lactone on paper chromatograms, using a mixture of the acid and lactone obtained by the hydrolysis of menthol-glucuronide according to the method of Quick (1927). It is clear (Partridge 1948, p. 243) that it was this mixture which was applied to the paper chromatograms, and which gave two spots corresponding respectively with glucuronic acid and glucuronolactone. However, also on p. 243 (Partridge 1948) it is stated that "glucuronic acid and galacturonic acid may be differentiated by the formation of the glucurone spot by the former," and on p. 248, in the discussion, it is said that "in acidic solvents solutions of glucuronic acid give rise to two well separated spots, one of them due to the lactone." It appears that there has been some misunderstanding due, firstly, to starting with a mixture of glucuronic acid and its lactone and secondly, to the formation of elongated spots by glucuronic acid which sometimes had "a marked forward trail." Both galacturonic and glucuronic acids give double or elongated spots in a number of solvent mixtures (Partridge 1948; Section III (*a*) (iii) above), and it has now been shown (Anet, Ash, and Reynolds, unpublished results) that the effect is due to certain of the cations in the paper.

Glucuronic acid ran slightly further than galacturonic acid in amyl alcohol-pyridine-water, but the acids ran the same distance in all of the other solvents tried, including ethyl acetate-acetic acid-water (Section III (*a*) (iii)). Whatman No. 1 and No. 4 papers were used in this work and the results for amyl alcohol-pyridine-water agreed with those obtained by Werner and Odin (1949) on Munktell OB paper; on the other hand Masamune and Maki (1952), using the same solvent mixture and Toyo No. 2 paper, recorded a good separation of these acids (R_F values 0·00 and 0·13). Rao, Beri, and Rao (1951) recorded R_F values of 0·17 and 0·30 for glucuronic and galacturonic acids on circular chromatograms run with ethyl acetate-acetic acid-water at 35°C, whereas in the present work these acids ran the same distance on descending chromatograms run at 20°C.

It appears therefore that galacturonic and glucuronic acids cannot at present be distinguished on paper chromatograms, and consequently it becomes necessary to elute the uronic acid from the paper and apply tests to the eluted acid as described in Section III (*c*).

(*b*) *Occurrence of Galacturonic Acid in Fruit*

A free uronic acid was detected by means of paper chromatography in several varieties of pears when the fruit was ripened at 20°C (Section III (*b*); Table 1; and Plate 1, Fig. 1); it was never found in the fruit as picked, although it may have been present in quantities not detectable under the conditions used. Where there was a variation in maturity at picking, the amount of uronic acid was related to the time of ripening (Table 1; Plate 1, Fig. 2).

A free uronic acid was also present in freestone peaches which had been ripened at 20°C, but was not detected in tree-ripened peaches (Table 1, samples 9-12). The free uronic acid was not detected in ripened clingstone peaches, nor in green or ripened apricots or apples (Table 1, samples 13-17*b*).

Chemical tests on some uronic acid eluted from paper chromatograms of pear extracts (Section III (*c*)) indicated that it was galacturonic acid. This was subsequently confirmed by other methods for peaches (Anet and Reynolds 1953) and pears (Anet and Reynolds, unpublished results).

The concentration of galacturonic acid in the sample which gave the strongest uronic acid spot (sample 2, Table 1) was 350 μg/g fresh fruit. The smallest amount of galacturonic acid which could be detected on paper chromatograms lay between 0·2 and 0·4 μg, corresponding with 30-60 μg/g fresh fruit. Most of the values obtained by earlier workers (Harris 1948; Winkler 1951, 1952; Mills 1952; cf. Section I above) were less than 60 μg/g.

Two significant points emerge from the present work. Firstly, a number of samples of pears and peaches ripened in a constant-temperature room at 20°C contained much higher concentrations of free galacturonic acid than any previously recorded. Secondly, where there was an appreciable variation in maturity at picking, the amount of free galacturonic acid present after ripening was greater in fruit which was originally less mature, and consequently required a longer time at 20°C to ripen.

The most probable mode of formation of free galacturonic acid in fruit would be by the enzymic degradation of pectin, which would require the presence of both pectin esterase and polygalacturonase (Lineweaver and Jansen 1951). Both of these enzymes can be produced by microorganisms, and pectin esterase is widely distributed in higher plants (Lineweaver and Jansen 1951; Kertesz 1951). However, Kertesz (1951) stated that, except in germinating barley, it was doubtful whether polygalacturonase occurred in higher plants. Since then Joslyn, Mist, and Lambert (1952), Weurman (1952), and Roelofsen (1953) have published the results of experiments which indicated the presence of polygalacturonase in apple juice, pears, and tomatoes respectively. Weurman (1952) also found that the pears studied (var. Doyenné Boussoc) contained a substance which acted as an inhibitor of polygalacturonase activity, and suggested that the occurrence of an inhibitor explained the failure of earlier attempts to establish the presence of polygalacturonase in fruits.

It therefore appears possible that the increased production of free galacturonic acid in fruit ripened at 20°C, now described, was due to a disturbance in the relationship between an inhibitor and an enzyme. On the other hand, pectic enzymes of fungal origin, if present, would become active when the fruit was transferred to 20°C. Most of the pear samples were ripened and extracted after the fruit had been stored for 4 or 5 months at – 1° or 0°C and this long storage period would increase the possibility of contamination by fungal enzymes. However, two pear samples were ripened and extracted after only 1 or 2 months storage, and none of the peach samples was cold-stored for more than a few days. Furthermore, there was no visible evidence of infection in the fruit used. It therefore seems unlikely that the results were due to the activity of fungal enzymes.

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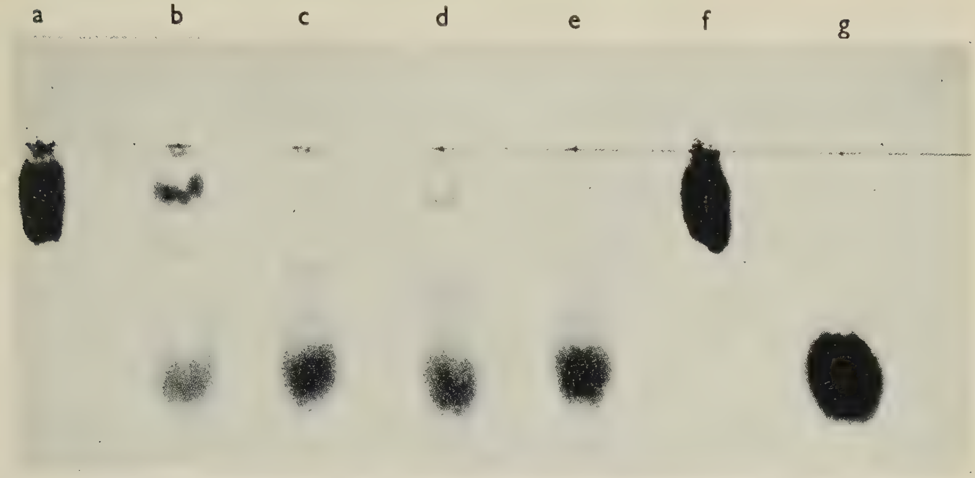
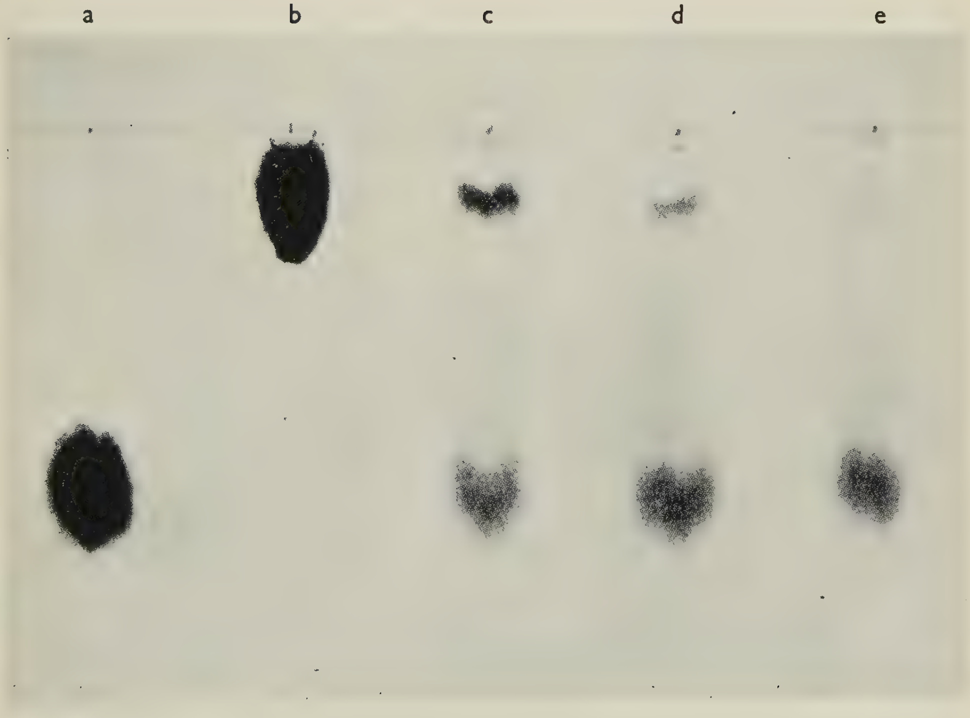
WATER-SOLUBLE CONSTITUENTS OF FRUIT. I





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Explanation of Plate 1

Fig. 1.—Portion of paper chromatogram on No. 1 Whatman paper (unwashed) run 24 hr in ethyl acetate-pyridine-water and treated with silver nitrate-sodium hydroxide showing starting line and

(*a*) D-Galacturonic acid;
(*b*) Galacturonic acid and inositol in Winter Cole pears (1951), from Batlow, N.S.W., ripened 13 days at 20°C (Table 1, 4*b*);
(*c*) Inositol in same lot of pears before ripening (Table 1, 4*a*);
(*d*) Galacturonic acid and inositol in Winter Cole pears (1951) from Orange, N.S.W., ripened 10 days at 20°C (Table 1, 3*b*);
(*e*) Inositol in same lot of pears before ripening (Table 1, 3*a*);
(*f*) D-Galacturonic acid;
(*g*) Meso-inositol.

Fig. 2.—Portion of paper chromatogram on No. 1 Whatman paper (unwashed) run 24 hr in ethyl acetate-pyridine-water and treated with silver nitrate-sodium hydroxide showing starting line and

(*a*) Meso-inositol;
(*b*) D-Galacturonic acid;
(*c*) Galacturonic acid and inositol in Packham's Triumph pears (1952), from N.S.W., ripened from green to ripe in 13 days at 20°C (Table 1, 7*c*);
(*d*) Galacturonic acid and inositol in same lot of pears ripened from medium to ripe in 8 days at 20°C (Table 1, 7*b*);
(*e*) Inositol in same lot of pears before ripening (Table 1, 7*a*).

as previously observed at the lower temperature, namely $K_s \approx 0{\cdot}2$. Additional comparisons were made using cultures of varying ages grown in aerated broth. As at the lower temperature, death rate at 50 per cent. R.H. was independent of the age of the culture. At 90 per cent. R.H., death rate and its temperature coefficient (Q_{10}) were both dependent on the age of the cells. At this relative humidity cells in the logarithmic phase had a Q_{10} of *c.* 2 whereas the value for old cells was *c.* 1. Supplementary figures showing values for K at 5°C are shown in Table 1.

Table 1

DEATH RATES AT 0, 5, 10, AND 15°C

Organism	Age (growth phase)	Relative Humidity (%)	K_d (hr^{-1})				
			0°*	5°	10°†	15°	
Esch. coli	Log phase	50	7·0	16	20	—	
		60	3·3	6·6	10·4	—	
		70	1·3	2·4	5·2	—	
		80	4·3	5·6	14·3	—	
Achromobacter (483)	Stationary phase	50	1·8	3·5	8·2	—	
		64	0·7	3·3	6·6	—	
Pseudomonas (451)	Stationary phase	51½	2·0	6·2	9·0	—	
		71	1·0	2·1	3·4	—	
			"Normal" Strain	"Sensitive" Strain	"Normal" Strain	"Normal" Strain	"Sensitive" Strain
Micrococcus (202)	Stationary phase	32				1·4-1·8	6
		68	0·5	—	5·8	10-14	40-50 (16)‡
		70-71	0·8	5·0	6·4	—	40-V. (12)‡ High
		76	1·8	—	5·2	—	25-30 (16)‡
		80	2·2	8·3	3·5	—	*c.* 27
		90	2·2	—	2·5	2·4-3·0	6-9

* Values from preceding papers (Brown 1953*a*, 1953*b*)

† Values from Figures 1 and 2.

‡ Values in brackets were obtained after a further period of 4 or 5 days incubation of the exposed petri dishes (i.e. a total incubation of 7 or 8 days at 20°C).

(*b*) *The Psychrophilic Genera*

The measurements made previously at 0°C with these three genera utilized cultures in their stationary phase of growth (Brown 1953*a*). This procedure was repeated at the higher temperatures and in addition the death rates of young (log phase) cells were measured. Death rates over complete growth cycles were not followed but rather it was sought merely to establish whether, in a given atmosphere, the log phase cells differed in sensitivity from old cells.

TABLE 2

INCIDENCE OF NON-LOGARITHMIC DEATH RATES OBSERVED WITH PSYCHROPHILIC GENERA AT 5, 10, AND 15°C

Organism	Number of Destruction Curves in Assessment	Number showing Non-Logarithmic Death	Incidence of Non-Logarithmic Death (%)
Achromobacter (483)	26	10	38·5
Micrococcus (202)	32	4	12·5
Pseudomonas (451)	19	3	15·8

(i) *Non-Logarithmic Death Rates.*—It was stated previously (Brown 1953*b*) that survivor curves of these strains were always logarithmic (at 0°C) in contrast with *Esch. coli* whose death rate sometimes deviated considerably from a simple logarithmic function. In the series of experiments to be described all three genera showed, on occasions, a high initial death rate followed by a gradual fall to a much lower rate. Routine streaking followed by subculture from a single colony seemed to eliminate the tendency toward non-logarithmic death, at least for a time. In *Micrococcus* 202, two strains of different sensitivities were isolated which separately gave logarithmic death rates of distinctly different magnitudes (Table 1). Table 2 shows the incidence of non-logarithmic

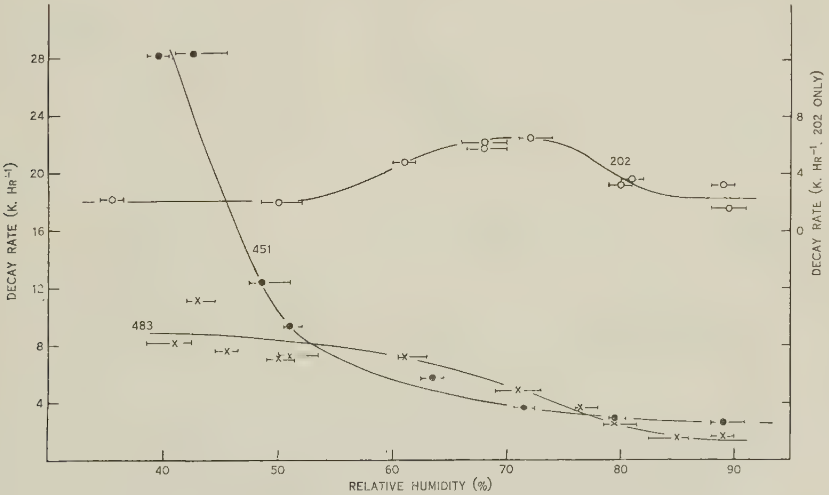

Fig. 2.—Decay rates (*K*) at 10°C. of *Achromobacter* 483 (×), *Micrococcus* 202 (O), and *Pseudomonas* 451 (●). Cultures were grown in standing tubes or bottles for 3 days at 20°C. The horizontal lines represent the range of humidities encountered for each measurement of *K*.

death rates in a number of measurements. This incidence might have been higher but for the routine precaution of preparing fresh stock cultures as soon as noticeable variations in colony size developed.

A feature of the "Sensitive" strain of 202 at 15°C is that, when sampled from an atmosphere of humidity in the neighbourhood of 70 per cent. R.H., colonies developing on nutrient agar after the usual incubation period of 3 days at 20°C, were always small (less than *c.* 0·5 mm dia.). Furthermore, colony size decreased with increasing time of exposure to the atmosphere, that is, colonies developing on late plates were smaller than those on the early ones. On further incubation the colonies grew to normal size and more colonies appeared. The new colonies were in greatest proportion on the later plates, resulting in a decrease in the apparent death rate (Table 1). This phenomenon was not observed to nearly the same extent at humidities around 40 and 90 per cent. where the colonies were almost entirely of normal size after the usual incubation period. Similarly the "Normal" strain of 202 usually gave colonies of normal size at all humidities and no decrease in death rate resulting from the appearance of more colonies on prolonged incubation. The significance of these observations is discussed later.

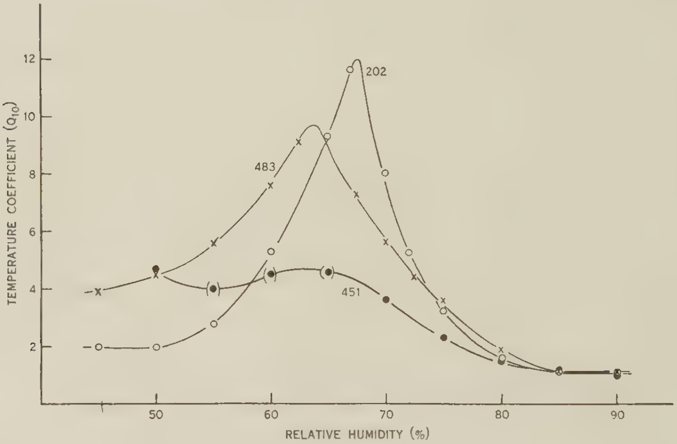

Fig. 3.—Temperature coefficients of K_d ($K_d = K - K_s$) for the range 0-10°C of *Achromobacter* 483 (×), *Micrococcus* 202 (O), and *Pseudomonas* 451 (●). Bracketed points for 451 correspond to the assumed points of the curve for this organism at 0°C (Brown 1953*a*).

(ii) *Effect of Temperature.*—Figure 2 illustrates the survival patterns at 10°C of the three strains. Figure 3 shows the temperature coefficients of the death rates related to relative humidity.

The results show several changes from those observed at 0°C. In all cases the minimum in the region of 65 per cent. R.H. has disappeared. There is, instead, for the *Achromobacter* a progressive increase in death rate with decreasing humidity, for the *Micrococcus* (202) a maximum at *c.* 70 per cent.

R.H., and for the *Pseudomonas* (451) a very sharp increase in death rate with decreasing humidity below about 60 per cent. R.H.

A superficial similarity in behaviour of the *Achromobacter, Micrococcus,* and *Esch. coli* lies in the occurrence of a maximum Q_{10} in the range 64-74 per cent. R.H. for each organism. This is the obvious consequence of the low death rates at 0°C near 70 per cent. R.H. With all organisms the death rates of old cells at 90 per cent. R.H. were independent of temperature. Values of K at 5° and 15°C are given in Table 1.

(iii) *Effect of Age.*—Comparisons of the death rates of cells, harvested during the logarithmic growth phase, were made with death rates of old cells at selected humidities. The results are summarized in Table 3. From this it will be seen that young cells of these three low-temperature organisms died more rapidly than old cells over the whole range of humidities employed. This behaviour is in contrast with that of *Esch. coli,* which displayed an age effect at high humidities only.

TABLE 3

EFFECT OF CULTURE AGE ON THE SURVIVAL OF THE PSYCHROPHILIC STRAINS AT 10°C

Organism	Method of Growth	Age and Stage of Growth	R.H. (%)	K (hr^{-1})
*Achromobacter** (483)	Aerated broth*	17 Hr*	53	42
	Standing tubed broth	3 Days (stationary phase)	53	8·2
	Standing tubed broth	16 Hr (early to mid log phase)	90	23
	Standing tubed broth	3 Days (stationary phase)	90	1·6
Micrococcus (202)	Aerated broth	17 Hr (mid to late log phase)	49	7·4
	Standing tubed and aerated broth	3 Days (stationary phase)	49	*c.* 2·1
	Aerated broth	17 Hr (mid to late log phase)	72	17
	Standing tubed and aerated broth	3 Days (stationary phase)	72	6·5
	Aerated broth	17 Hr (mid to late log phase)	89	6·9
	Standing tubed and aerated broth	3 Days (stationary phase)	89	1·6-3·2
Pseudomonas (451)	Aerated broth	17 Hr (mid log phase)	52	*c.* 43
	Standing tubed broth	3 Days (stationary phase)	52	10
	Standing tubed broth	17 Hr (early to mid log phase)	89	17
	Standing tubed broth	3 Days (stationary phase)	89	2·6

* When grown in aerated culture 483 lysed (with 50-75 per cent. reduction in turbidity) at approximately this stage. Growth was resumed some hours after lysis. Similar values for K were obtained just before and just after lysis.

(*c*) *Effect of Sodium Chloride*

Dunklin and Puck (1948) have claimed for several airborne pathogenic cocci a lethal action of sodium chloride in the vicinity of 50 per cent. R.H. A comparison was made of their results with the behaviour of three of the organisms of the present study which were sprayed at representative humidities from suspension in sodium chloride solution (0·9 per cent. w/v). The basic observations of Dunklin and Puck were confirmed for these organisms, as no effect of sodium chloride on death rates was apparent at high humidities but in all cases increases were observed near 50 per cent. (Table 4).

TABLE 4

EFFECT OF SODIUM CHLORIDE ON DEATH RATES OF BACTERIA AIRBORNE AT 10°C

Organism	Relative Humidity (%)	K_d (hr^{-1})	
		Suspension in Water	Suspension in Physiological Saline
Esch. coli	46	20-24	>40
	50	13-16	*c.* 23
	80-81	*c.* 14	8·0-8·8*
Achromobacter (483)	47-50	7·2-8·4	11-14
	82	2·0-2·2	2·3
Micrococcus (202)	47	*c.* 2·0	13
	68	6·2	5·8
	87	*c.* 2·6	2·8

* These cultures were slightly older than their controls.

IV. DISCUSSION

The results presented in this paper indicate that substantial differences may exist between survival patterns of airborne bacteria at 0 and 10°C. In addition, at the higher temperature, the differences between individual strains of bacteria have been emphasized. The results obtained with *Esch. coli* lend support to the proposal, made on the basis of the observations at 0°C, that at least two distinct lethal mechanisms may exist at high and low humidities. On the other hand, the results for the low-temperature organisms do not clearly support this contention. The results for the *Micrococcus* actually seem to provide evidence against such a proposal.

The comparison of death rates at different temperatures on the basis of relative humidity is, in itself, accompanied by some dangers. Shifts in the relation, K/R.H., do occur and, apart from the effects on calculated values of Q_{10}, such shifts may confuse the general interpretation of results.

The occasional observation of non-logarithmic survivor curves at 0 and 10°C, and the conditions under which these occurred, suggest that such curves may result from heterogeneity in the bacterial suspensions used (cf. Dunklin and Puck 1948; Mitchell 1951; Eddy and Hinshelwood 1953; Eddy 1953*a*, 1953*b*). Some support is given to this belief by the isolation of two strains of the *Micro-*

coccus (Table 1). The slower rate of development of colonies of the "sensitive" strain of the *Micrococcus* on nutrient agar may be indicative of progressive damage to the airborne cell occurring prior to death. In the light of a possibility of this kind it is well to realize that sampling with the "Slit-Sampler" technique does not distinguish between damage occurring while the cell is airborne and that which may occur during the rapid re-hydration taking place on sampling onto the nutrient medium. Damage during re-hydration may well be related to the history of a cell up to the time of sampling and, if so, could affect the measured value of a "death rate." It is apparent that techniques in addition to those used already will be needed to provide information about problems of this kind.

V. ACKNOWLEDGMENT

It is a pleasure to acknowledge the technical assistance and general participation in this programme of Mr. F. Grau.

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BACTERIAL OXIDATION OF ARSENITE

I. DESCRIPTION OF BACTERIA ISOLATED FROM ARSENICAL CATTLE-DIPPING FLUIDS

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Summary

From arsenical cattle-dipping fluids from Queensland, in which spontaneous oxidation of arsenite to arsenate had occurred, 15 strains of bacteria were isolated which are able to bring about oxidation of arsenite in culture media. Their characteristics, their taxonomy, and some factors which influence their arsenite-oxidizing activity are described. The strains share many common features, but do not form a homogeneous group. They are regarded as constituting five species, of which three belong to the genus *Pseudomonas*, and one to the genus *Xanthomonas*, in the family Pseudomonadaceae; the remaining species is allotted to the genus *Achromobacter*, in the family Achromobacteriaceae.

I. Introduction

Spontaneous oxidation of arsenite to arsenate in cattle-dipping fluids was first described by Brünnich (1909) in Queensland. It was subsequently reported in the U.S.A. by Fuller (1911) and Chapin (1915), and in South Africa by Williams (1913). The observations of those workers, and by Brünnich and Smith (1914), that it could be prevented or arrested by disinfectants of the coal-tar series, suggested a causal relationship with bacterial growth. Finally, at Onderstepoort, South Africa, Green (1918*a*) isolated from a dipping fluid a bacterium, provisionally named *Bacillus arsenoxydans,* which in pure cultures was able to bring about oxidation of arsenite to arsenate. Appearing in a yearly publication mainly devoted to animal diseases, this important and detailed work apparently escaped the notice of general microbiologists. The bacterium was eventually lost, and it is therefore no longer available for study. This appears to be the only record of arsenite-oxidizing bacteria until the author's preliminary note (Turner 1949).

In 1944, when the investigations to be described were begun, the cause of spontaneous oxidation of arsenical cattle-dipping fluids in Queensland had not been determined, although empirical methods existed for overcoming it (see Turner 1943). It was therefore decided to examine partly "oxidized" cattle-dipping fluids from Queensland for the presence of arsenite-oxidizing bacteria. If they were found, it was hoped that the study of their characteristics and mode of action might lead to more specific means of controlling the deterioration. Eventually, 15 strains of arsenite-oxidizing bacteria were isolated, and their general features were briefly described in the preliminary note referred to.

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A more complete description of the bacteria, and of the enzymic mechanism concerned in oxidation of arsenite, is now presented in a series of papers.

This first paper of the series describes the isolation of arsenite-oxidizing bacteria from oxidized arsenical cattle-dipping fluids from various parts of Queensland, and discusses their characteristics, their taxonomy, and some factors which influence their activity.

II. METHODS

(*a*) *Temperature of Incubation*

Unless otherwise stated, 25°C.

(*b*) *Enrichment Media*

In the early experiments, the enrichment medium of Green (1918*a*) was used. For the main part, a medium referred to briefly as KA3 was devised. This consists of the citrate-ammonium medium of Koser (1923), modified by the addition of 0·26 per cent. (=0·02M) sodium meta-arsenite; it is adjusted to pH 8·1 before autoclaving, and is moderately buffered over the range pH 4·5 to 7·5 by the phosphoric and citric acid systems.

(*c*) *Isolation Procedure*

One ml of the sample was sown into 200 ml of KA3 in a 500-ml erlenmeyer flask. Progress of oxidation was followed by means of iodometric titration. When oxidation was well advanced, or was complete, the enriched cultures were stroked on nutrient agar, usually without a second enrichment. Representative colonies were sown into a presumptive test medium, by means of which arsenite oxidizers were quickly recognized. It consists of KA3 with 0·0032 per cent. bromothymol blue as indicator, adjusted to pH 7·0, and distributed in 5-ml amounts in 6 by ⅝ in. test-tubes. Inoculated tubes were incubated almost horizontally, in order to increase the surface area. The test is based upon the fall of pH which accompanies the oxidation of arsenious acid ($pK = 9{\cdot}22$) to arsenic acid ($pK_1 = 2{\cdot}3$); oxidation of arsenite is accompanied by a change from faint blue to yellow, whereas strains not able to oxidize arsenite usually cause a deepening of the blue tint.

(*d*) *Fermentation Tests*

These were carried out in peptone solution, adjusted to pH 7·0 and containing 1 per cent. Andrade's indicator; 1 per cent. of test substance, separately sterilized by filtration, was added aseptically. Incubation was continued for 21 days. Micro-fermentation tests were also carried out by the procedure of Cowan (1953), modified by the inclusion of 0·017M phosphate buffer pH 7·4. Cell suspensions for this were harvested from young cultures on Cotton's potato-infusion beef-extract glucose glycerol peptone agar (PA), prepared as described by Mitchell and Moore (1941), which gives a high yield of cells. Some tests were also carried out by the procedure of Hugh and Leifson (1953), in order to determine the type of attack on carbohydrates, i.e. whether oxidative or fermentative.

(*e*) *Carbon-source Utilization*

The basal medium of Seleen and Stark (1943), adjusted to pH 7·0, was used. Growth was judged by opacity, and acid production was determined by adding a drop of bromocresol purple solution. Incubation was continued for 25 days.

(*f*) *Serological Tests*

Antisera were prepared in rabbits. Agglutination tests, carried out with thick suspension on glass plates, were read within 2 min. Antigens for precipitin tests were prepared by modification of Lancefield's method described by Stableforth (1932).

(*g*) *Estimation of Arsenite Oxidation*

Arsenite was estimated by iodometric titration. The $AgNO_3$ spot test for arsenate (Feigl 1937) was used as a qualitative test for oxidation, but in dipping fluids contaminated with excreta, or in cultures in KA3, a positive reaction was not obtained until oxidation had proceeded to the extent of 30-50 per cent.

III. Results

(*a*) *Isolation of the Strains*

From each of the 15 samples bacteria were isolated which were capable of bringing about oxidation of arsenite to arsenate in suitable culture media. It is assumed that they were responsible for the oxidation of the respective dipping fluids. In the early stages of investigation it was found that samples from the dip at the Cannon Hill Meatworks, Brisbane, yielded only one type of arsenite-oxidizing bacterium which formed on nutrient agar very characteristic colonies. Its unusual appearance was used in recognizing it in a total of 10 samples. It is assumed that it was the only arsenite-oxidizing type present, because all other colonies examined failed to bring about oxidation of arsenite. However, in five samples this bacterium, provisionally referred to as type I, was not present. This led to the recognition of types II, III, IV, and V, of which type II was recovered on two occasions; but in none of the samples was more than the one type found.

(*b*) *Source and Geographical Distribution of the Strains*

The samples from which the strains were isolated were allotted serial numbers when received. The date when collected, the location of the dip, the provisional "type" to which the isolated strain was allotted, and the suggested classification and nomenclature of the strains, are set out in Table 1.

In Queensland, October and December correspond to the warmer period of the year and the early "wet season"; June corresponds to the relatively cooler period of the year.

The geographical distribution is clearer from the map (Fig. 1). Type I was found widely between lat. 21° S. and 28° S.; types II and III occurred on the coast near the Tropic of Capricorn; and types IV and V were found under

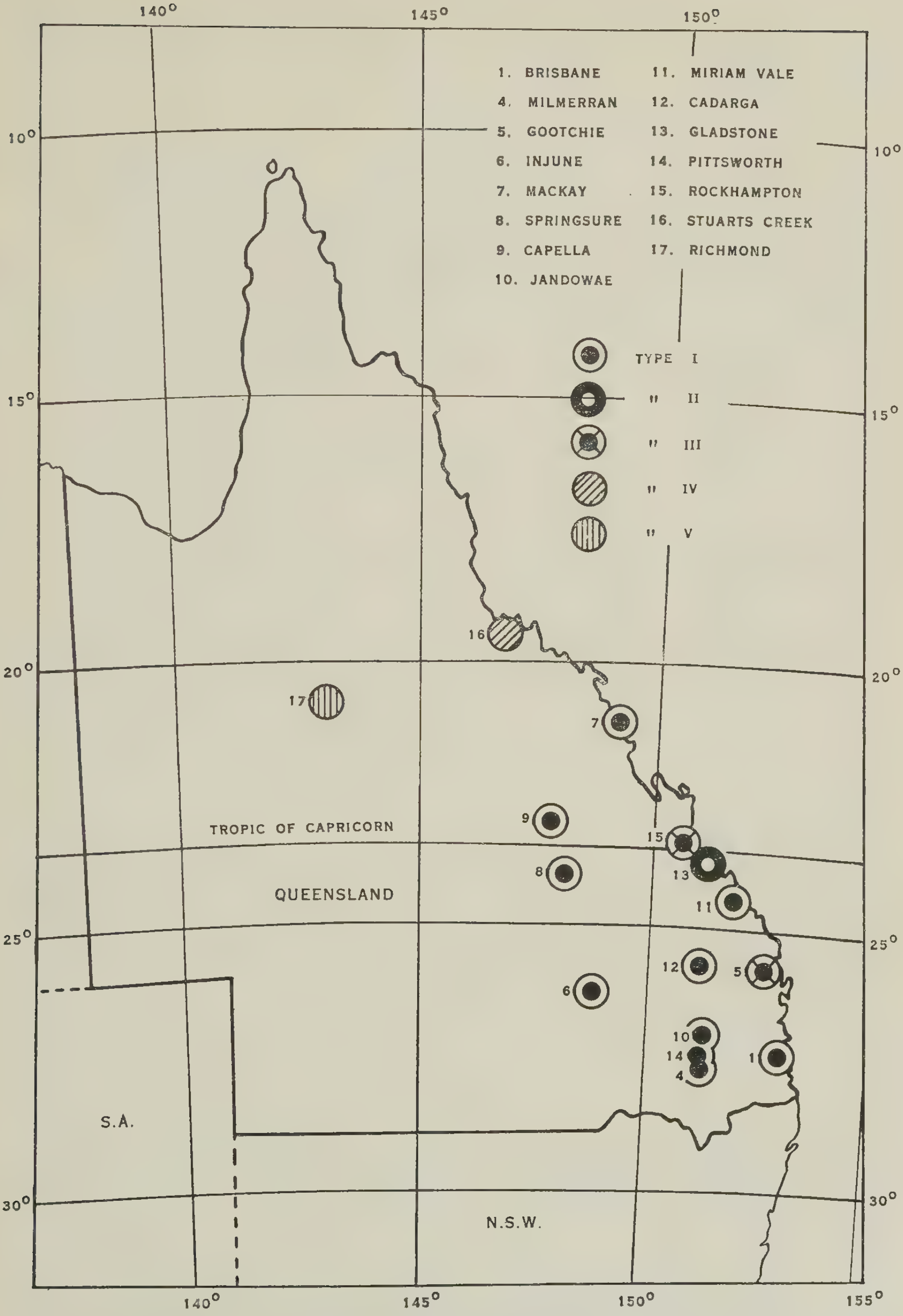

Fig. 1.—Map of Queensland, showing location of the arsenical cattle dips from which samples were obtained, and the provisional "type" to which the strains isolated were assigned.

warm conditions near lat. 20° S., type IV near the coast and type V inland. However, in view of the small number of samples, the distribution may not be significant.

Table 1

PARTICULARS OF ARSENITE-OXIDIZING BACTERIA ISOLATED FROM QUEENSLAND ARSENICAL CATTLE-DIPPING FLUIDS

Strain No.	Date Collected	Locality	Provisional "Type"	Designation
1, 2, 3	3.x.1944	Brisbane	I	*Ps. arsenoxydans-unus* sp. nov.
4	3.x.1944	Milmerran	I	*Ps. arsenoxydans-unus* sp. nov.
6	5.vi.1945	Injune	I	*Ps. arsenoxydans-unus* sp. nov.
7	5.vi.1945	Mackay	I	*Ps. arsenoxydans-unus* sp. nov.
8	5.vi.1945	Springsure	I	*Ps. arsenoxydans-unus* sp. nov.
9	5.vi.1945	Capella	I	*Ps. arsenoxydans-unus* sp. nov.
10	5.vi.1945	Jandowae	I	*Ps. arsenoxydans-unus* sp. nov.
11	5.vi.1945	Miriam Vale	I	*Ps. arsenoxydans-unus* sp. nov.
12	5.vi.1945	Cadarga	I	*Ps. arsenoxydans-unus* sp. nov.
14	5.vi.1945	Pittsworth	I	*Ps. arsenoxydans-unus* sp. nov.
5	5.vi.1945	Gootchie	II	*Ps. arsenoxydans-duo* sp. nov.
15	5.vi.1945	Rockhampton	II	*Ps. arsenoxydans-duo* sp. nov.
13	5.vi.1945	Gladstone	III	*Achr. arsenoxydans-tres* sp. nov.
16	8.xii.1947	Stuart's Creek	IV	*Xanth. arsenoxydans-quattuor* sp. nov.
17	8.xii.1947	Richmond	V	*Ps. arsenoxydans-quinque* sp. nov.

(c) *Description of the Bacteria*

(i) *Common Features*

Although these bacteria may be separated into several types, they have many similarities. They are all Gram-negative, non-sporulating, aerobic, motile rods. They grow well in the presence of bile salts or of penicillin. They do not produce gas from any attackable carbohydrate; those that attack carbohydrates do this oxidatively and not fermentatively. They do not produce acid from lactose, nor can they utilize it as the sole source of carbon. They do not produce acetylmethylcarbinol. They break down nitrates, produce small amounts of H_2S but not indole, and are catalase-positive. They have a slight, delayed alkalinizing action upon milk. They do not produce turbidity in lecithovitellin solution. They are able to grow well on simple culture media such as Koser's medium, in which the source of nitrogen is the NH_4^+ ion and the source of carbon is citrate or other simple organic source. None grew in the inorganic basal medium of Doudoroff (1940), in which was incorporated 0·02M $NaAsO_2$, when placed in an atmosphere of 80 per cent. CO_2 and 20 per cent. O_2. There was thus no evidence from this experiment to suggest that they can use the energy of oxidation of arsenite for growth, but further investigation is desirable. They grow very luxuriantly on the usual meat-infusion, peptone, or yeast-extract types of culture media, and are therefore typical heterotrophic microorganisms. They appear to be non-pathogenic to animals: young active cultures in meat-infusion peptone broth did not produce ill effects when injected intramuscularly into guinea pigs in a dose of 1 ml, nor intraperitoneally into mice in a dose of 0·25 ml. None of the strains is haemolytic when grown on the surface of sheep-blood meat-infusion peptone agar.

(ii) *Differential Features*

The differentiation of these strains into "types" was based upon the following characteristics: cell size, type of flagellation, colony form, pigment production, ability to grow anaerobically or autotrophically, temperature requirements for growth, serological reactions, biochemical properties (including pigment production), proteolytic ability, ability to produce acid from the usual range of "fermentation" substances, and ability to utilize various compounds as the sole source of carbon.

Cell size.—The cells of strains 5 and 15 (type II) are larger, particularly in width, than those of the other strains when grown on the same medium such as PA.

Type of flagellation.—Types I, II, IV, and V develop polar flagella, either singly or in tufts, whereas strain 13 (type III) is peritrichially flagellated.

Colony form.—On the surface of infusion peptone agar or of PA, the strains classed as type I form colonies which at first are whitish by reflected light, but develop semi-translucent dirty greyish flecks or streaks; they are very soft and semi-fluid, and readily coalesce to form very large colonies. If cultures in petri dishes are left inverted, the colonies may drip to the lower lid or may run down the side under the influence of gravity. Strain 16 (type IV) also is quite distinctive: its colonies are tough and gelatinous, and they are much wrinkled, more or less concentrically; when more thickly sown, the continuous surface growth is thrown into great irregular folds. Types II, III, and V are less distinctive.

Pigment production.—Strains 5 and 15 (type II) quickly produce strongly fluorescent greenish yellow soluble pigment in most media, and particularly well in the medium of Georgia and Poe (1931). On this medium, the strains grouped as types I, III, IV, and V do not produce pigment. Strain 16 (type IV) produces bright yellowish brown pigmented growth on potato.

Temperature requirements.—Strains 5 and 15 (type II) grow relatively well at low temperatures (3°C), but not at all at 37°C, whereas all other strains fail to grow at 3°C even after 4 wk, but will grow at 37°C. Strain 16 (type IV) is unique, in that it will grow infusion peptone broth even at 44°C.

Fermentation reactions.—Strains 1, 4, 5, 6, 7, 10, 11, 12, 13, 14, and 15 were subjected to these tests in the usual way, with peptone solution as the basal medium; strains 16 and 17, which were isolated subsequently, were not. It was difficult to obtain reproducible results, and it was assumed that production of alkali obscured acidifaction. Similar difficulties with standard fermentation tests, because of concurrent ammonification, have been recorded by many investigators (e.g. Stein, Weaver, and Scherago 1942; Orcutt 1943; Seleen and Stark 1943). The main findings were as follows. None of the strains produced gas; those subsequently grouped under type I (i.e. strains* 1, 4, 5, 6, 7, 10, 11, and 12) all produced an acid reaction with xylose, some with arabinose and rhamnose, some with glucose and fructose, some with erythritol and adonitol, but none with any other member of the wide range of test substances; those grouped under type II (strains 5 and 15) produced an acid reaction with xylose, arabinose, glucose, and fructose only; and type III (strain 13) did not produce an acid reaction with any test substance.

Tests for preformed or readily synthesized enzymes were carried out by the method of Cowan (1953). Although cells harvested from any single medium, such as PA, may not attack all the substrates which the bacterium is capable of attacking, the findings under the defined conditions help to differentiate the types. Types III and V were unable to produce acid under these conditions from any of the extensive range of pentoses, hexoses, disaccharides, trisaccharides, polysaccharides, and alcohols. Types I, II, and IV produced acid from the pentoses arabinose and xylose, but not from rhamnose. Acid was produced from the hexoses as follows: type I—glucose and fructose; type II—glucose, mannose, and galactose; type IV—glucose, fructose, mannose, and galactose. Acid was not produced by any of the strains from the trisaccharide raffinose, or from the polysaccharides (starch, inulin, dextrin, glycogen). Of the disaccharides (saccharose, maltose, lactose, trehalose) the only one to yield acid under these conditions was maltose, with type II. Of the alcohols (methanol, ethanol, glycerol, erythritol, adonitol, mannitol, dulcitol, sorbitol) only ethanol and dulcitol yielded acid, again with type II. The glucosides aesculin and amygdalin, but not salicin, yielded acid with type II and with type IV. The non-carbohydrate inositol did not yield acid with any type.

Tests by the procedure of Hugh and Leifson (1953), in order to determine the mode of attack upon xylose and on glucose, showed that, when it occurred, it was strictly oxidative. Types III and V were without action; types I, II, and IV oxidatively assimilated both sugars, the attack upon glucose by type I being only slight under those conditions.

Utilization of carbon compounds as a sole C source.—The first series of tests (Table 2) comprised the extensive list of substances used in the fermentation tests, together with two phosphorylated hexoses, glucose-1-phosphate and fructose-1, 6-diphosphate.

Some distinctive features were as follows:

Type I utilized all three pentoses, all the alcohols, and inositol.

Type II could not utilize any alcohol higher than the trihydric glycerol.

Type III was distinctive in its relative inertness towards these substances; it could not utilize any saccharide, or any alcohol higher than ethanol.

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* Strains 2, 3, and 8 also belonged to this type, but were lost before complete tests could be made.

Type IV utilized starch and glycogen.

Type V could not utilize any hexose, although it utilized maltose and raffinose.

TABLE 2

CARBON SOURCE UTILIZATION

(*a*) "Fermentation-test" series

Substance	Type I								Type II		Type III	Type IV	Type V
	Strain								Strain				
	1	4	6	7	10	11	12	14	5	15	13	16	17
Arabinose	++	++	++	++	++	++	++	++	++	++	−	−	−
Xylose	++	++	++	++	++	++	++	+	++	++	−	++	+
Rhamnose	++	++	++	++	++	++	++	++	−	−	−	−	−
Glucose	++	++	++	++	++	++	++	++	++	++	−	++	−
Fructose	+	++	++	++	++	++	++	+	++	++	−	++	−
Mannose	++	++	++	++	+	++	+	++	−	−	−	−	−
Galactose	++	++	++	++	++	++	++	++	++	++	−	++	−
Sucrose	+	+	+	+	−	+	−	−	−	−	−	−	−
Maltose	+	+	−	+	+	+	++	+	+	+	−	++	+
Lactose	−	−	−	−	−	−	−	−	−	−	−	−	−
Raffinose	−	−	−	−	−	−	−	−	−	−	−	−	+
Starch	−	−	−	−	−	−	−	−	−	−	−	++	−
Inulin	+	++	+	+	+	+	+	+	+	+	++	−	+
Dextrin	+	+	+	+	+	+	+	+	+	+	−	++	+
Glycogen	−	−	−	−	−	−	−	−	−	−	−	++	−
Methanol	+	+	+	+	+	+	+	+	+	+	+	+	+
Ethanol	++	++	+	+	+	++	++	++	++	++	++	++	++
Glycerol	+	+	+	+	+	+	+	+	++	++	−	++	++
Erythritol	++	++	+	++	+	++	+	+	−	−	−	−	−
Adonitol	++	++	++	++	++	++	++	++	−	−	−	−	−
Mannitol	+	+	+	+	+	+	+	−	−	−	−	++	+
Dulcitol	+	+	+	+	+	+	+	+	−	−	−	−	−
Sorbitol	+	+	+	+	+	+	+	+	−	−	−	−	−
Amygdalin	+	+	+	+	+	+	+	+	+	+	+	+	+
Salicin	−	−	−	−	−	−	−	+	−	−	−	−	−
Inositol	+	+	+	+	+	++	+	+	−	−	−	−	−
l-Sorbose	+	+	−	−	−	−	−	+	−	−	−	−	−
Trehalose	+	+	−	−	−	+	+	+	−	−	−	−	−
Glucose-1-phosphate	+	+	+	+	+	+	+	+	++	++	−	+	+
Fructose-1, 6-diphosphate	+	+	+	+	+	+	+	+	+	+	+	+	+

+ = Weak growth, ++ = strong growth.

The second series of tests (Table 3) included various aliphatic, aromatic, cyclic, and heterocyclic compounds that were available, but it was not possible to carry out systematic tests upon homologous series.

Table 3

CARBON SOURCE UTILIZATION

(*b*) Miscellaneous series

Test Substance	Concentration (%)	Type I								Type II		Type III	Type IV	Type V
		1	4	6	7	10	11	12	14	5	15	13	16	17
Formic acid	1·0	+	+	−	+	+	+	+	+	+	+	+	+	+
Acetic acid	1·0	++	++	++	++	++	++	++	++	++	++	++	++	++
Lactic acid	1·0	++	++	++	++	++	++	+	++	++	++	++	++	++
Oxalic acid	1·0	+	+	+	±	±	±	±	+	+	±	+	+	+
Mucic acid	1·0	+	+	+	+	+	±	+	+	+	+	−	−	++
Tartaric acid	1·0	+	+	+	+	+	+	+	+	++	+	+	+	++
Ascorbic acid	1·0	+	+	+	+	+	+	+	+	±	±	−	+	−
Glycollic acid	1·0	+	+	+	+	+	+	+	±	+	±	++	+	±
Na glycollate starch ether	1·0	+	+	+	+	+	+	+	±	+	±	+	+	±
Succinic acid	1·0	+	+	+	+	+	+	+	+	++	++	+	++	++
Fumaric acid	0·25	++	+	+	+	+	+	+	+	++	++	+	++	++
Malic acid	0·25	+	+	+	+	+	+	+	+	++	++	++	++	++
Aconitic acid	0·25	+	+	+	+	+	+	+	+	++	++	+	+	+
Pyruvic acid	0·5	+	+	+	+	+	+	+	+	++	++	+	++	++
Mandelic acid	0·5	+	+	+	+	+	+	+	+	+	±	+	±	0
Adenylic acid	0·25	+	+	+	+	+	+	+	+	+	+	+	+	+
Butyric acid	0·5	−	−	−	−	−	−	−	−	++	++	++	−	−
Phenol	0·05	+	+	+	+	+	+	+	+	++	++	−	+	+
p-Hydroxyphenyl-acetic acid	0·05	+	+	+	+	+	+	+	+	++	++	+	+	++
Benzoic acid	0·1	−	−	−	−	−	−	−	−	++	++	+	−	−
p-Hydroxybenzoic acid	0·05	+	+	+	+	+	+	+	+	++	++	+	−	±
p-Aminobenzoic acid	0·05	+	+	+	+	+	+	+	+	−	−	+	−	++
o-Aminobenzoic acid	0·05	−	−	−	−	−	−	−	−	−	−	−	−	−
Salicylic acid	0·05	−	−	−	−	−	−	−	−	−	−	−	−	−
p-Cresol	0·05	−	−	−	−	−	−	−	−	++	++	−	−	−
Aniline	0·05	+	+	+	+	+	+	+	+	±	±	±	+	±
Pyridine	0·05	+	+	+	+	+	+	+	+	±	±	+	−	+
α-Picoline	0·05	+	+	+	+	+	+	+	+	±	±	+	−	+
s-Collidine	0·05	+	+	+	+	+	+	+	+	+	−	+	−	+
Pimelic acid	0·05	+	+	+	+	+	+	+	+	+	+	+	±	++
Phthalic acid	0·05	+	+	+	+	+	+	+	±	+	±	+	+	+
Diethyleneglycol	1·0	+	+	+	+	+	+	+	+	±	+	+	+	+

TABLE 3 (*Continued*)

β-Glycerosphosphoric acid	1·0	+	+	+	+	+	+	+	+	+	+	+	+	+
Sodium luminal	0·1	+	+	+	+	+	+	+	+	+	+	+	+	+
Phenylpropionic acid	0·05	−	−	−	−	±	−	−	−	±	±	−	±	±
Uric acid	0·05	+	+	+	+	+	+	+	+	+	+	−	+	+
α-Naphthylacetic acid	0·03	±	±	−	−	−	−	−	−	+	+	−	+	+
*cyclo*Hexanol	0·05	+	+	+	+	−	+	+	+	−	−	+	−	−
Methyl-*cyclo*-hexanone	0·02	+	+	+	+	+	+	+	+	+	+	+	+	+

± = Very slight growth, + = moderate growth, ++ = strong growth, 0 = not done.

Some distinctive features were as follows:

Type II was the most versatile and utilized even *p*-cresol.

Type III could not utilize uric acid or phenol.

Type IV was the least versatile; it could not utilize any of the pyridine or benzoic series but, like types II and V, could utilize phenol.

Type V, like types I and IV, could not utilize butyric acid.

Proteolytic properties.—Strain 16 alone, classed as type IV, liquefies gelatine and also inspissated serum. Strain 17 (type V) causes very slight softening of gelatine, but not liquefaction, near the line of inoculation, after many weeks' incubation at 25°C. The others are completely without action.

Action upon nitrate.—All the strains break down nitrate, but whereas types I, IV, and V produce gas, presumably N_2, N_2O, or both, types II and III reduce it still further to NH_3.

Ability to grow autotrophically.—Types I and II are probably facultative chemosynthetic autotrophs, able to obtain energy for growth by oxidizing H_2 to water when more complex compounds are not available. In an unrepeated experiment, these types grew slowly, whereas types III, IV, and V did not, in the simple inorganic solution of Doudoroff (1940) containing NH_4^+ ion as the N source, when placed in an atmosphere of H_2 (83 per cent.), CO_2 (15 per cent.), and O_2 (2 per cent.).

Serological relationships.—The results of the agglutination tests are set out in Table 4, in which the strains are arranged according to the provisional types. It is evident that (*a*) within type I, strains 1, 4, 11, and 12 form a fairly homogeneous subtype, the other strains constituting distinctive or individual subtypes; (*b*) types II, III, IV, and V are sharply distinguished from each other and from type I; and (*d*) strains 18*L* and 18*S*[*], isolated from cattle faeces in Melbourne, are antigenically distinctive, but have some cross relationships with types II and V respectively. The agglutinin reactions were confirmed to a con-

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[*] These strains have not been examined otherwise in detail.

TABLE 4

AGGLUTININ AND PRECIPITIN RELATIONSHIPS OF ARSENITE-OXIDIZING BACTERIA

Antigens		Antisera														
Type	Strain	Type I								Type II		Type III	Type IV	Type V	Melbourne Types	
		1	4	11	12	10	6	7	14	5	15	13	16	17	18*L*	18*S*
I	1	9(+)	9(+)	9(+)	7(+)	−(−)	−(−)	−(−)	1(−)	−(−)	−(−)	−(−)	—	−(−)	−(−)	−(−)
	4	10(+)	10(+)	10(+)	7(+)	−(−)	−(−)	−(−)	−(−)	−(−)	−(−)	−(−)	—	−(−)	−(−)	−(−)
	11	10(+)	10(+)	10(+)	6(+)	3(−)	2(−)	3(−)	1(−)	−(−)	−(−)	−(−)	—	−(−)	1(−)	1(−)
	12	9(±)	9(±)	9(±)	8(+)	−(+)	−(+)	−(+)	−(+)	−(−)	−(−)	−(−)	—	−(−)	−(−)	−(−)
	10	4(−)	3(−)	4(−)	7(−)	9(+)	2(−)	9(−)	4(−)	−(−)	−(−)	−(−)	—	−(−)	2(−)	2(−)
	6	1(±)	1(±)	−(±)	2(+)	1(+)	7(+)	−(+)	−(+)	−(−)	−(−)	−(−)	—	−(−)	−(−)	−(−)
	7	−(−)	−(−)	−(−)	−(−)	−(−)	−(−)	8(+)	−(−)	−(−)	−(−)	−(−)	—	−(−)	−(−)	−(−)
	14	4(−)	4(−)	5(−)	5(+)	−(−)	−(+)	−(−)	9(+)	−(−)	−(−)	−(−)	—	−(−)	1(−)	1(−)
II	5	−(−)	−(−)	−(−)	5(−)	−(−)	−(−)	−(−)	−(−)	10(+)	10(+)	−(−)	—	−(−)	3(−)	3(−)
	15	−(−)	−(−)	−(−)	2(−)	−(−)	−(−)	−(−)	−(−)	9(+)	9(+)	−(−)	—	−(−)	1(−)	1(−)
III	13	−(−)	−(−)	−(−)	−(−)	−(−)	−(−)	−(−)	−(−)	−(−)	−(−)	17(+)	—	−(−)	2(−)	2(−)
IV	16	—	—	−(−)	−(−)	—	−(−)	—	−(−)	—	—	−(−)	5	—	—	—
V	17	−(−)	−(−)	−(−)	2(−)	−(−)	−(−)	−(−)	−(−)	−(−)	−(−)	−(−)	—	9(+)	9(±)	9(+)
Melbourne types	18*L*	2(−)	2(−)	−(−)	−(−)	−(−)	−(−)	−(−)	−(−)	6(+)	6(+)	−(−)	—	−(−)	8(+)	4(+)
	18*S*	−(−)	−(−)	−(−)	−(−)	−(−)	−(−)	−(−)	−(−)	1(−)	1(−)	−(−)	—	10(+)	11(+)	11(+)

The agglutinin titres are expressed as $\log_2$ (titre/10): thus $2 \equiv 20$, $10 \equiv 5120$, etc. The symbols in parentheses refer to the precipitin reaction: + = positive, ± = weak, − = negative.

siderable extent by the precipitin reactions, indicating that, in general, the same antigenic components are implicated in the two reactions, although some inconsistencies suggest that this is not so in all strains.

Rate of arsenite oxidation in KA3 medium.—During prolonged subculturing, frequently from single colonies, arsenite-oxidizing activity of types II and V decreased to some extent. This was correlated with an increase of cell size in type V, and appears to have been the result of chance selection of mutants. By suitable re-selection of colonies, full activity and normal morphology were restored to type V. Type II used to bring about complete oxidation of 0·02M arsenite in KA3 within 4 days, but now requires about 7 days; possibly re-selection of colonies could restore the original activity.

The present position is summed up in Figure 2, which gives the oxidation curves of the type strains of the five types. The most active is type V.

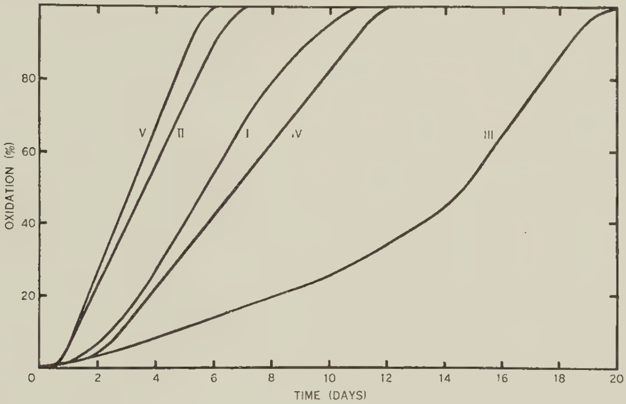

Fig. 2.—Arsenite oxidation curves of the "type strains" of the five species, when incubated with KA3 medium at 25°C.

The differences in arsenite-oxidizing activity in KA3 at 25°C are probably due partly to differences in growth rate. This has not been studied in detail, but it was found that equally dense washed cell suspensions of types I, III, IV, and V harvested from KA3 cultures were equally active when shaken with arsenite.

Influence of temperature upon arsenite oxidation.—Interesting differences in arsenite-oxidizing ability of the types were revealed when incubation was carried out at 37°C instead of at 25°C. Type II will not grow at 37°C, even in nutritionally complex media, but the other four types grow quite satisfactorily. Under these conditions, type I was slightly less active than at 25°C, and required 14 days instead of 11 days for complex oxidation; on the other hand, type III was slightly more active, and at 37°C required 16 days instead of 20 days at 25°C. Types IV and V grew well at 37°C, but failed to oxidize arsenite. Tests with type IV, which is capable of growth even at 44°C, suggested that this is due to temperature sensitivity of the enzyme system: cells harvested from KA3 cultures grown at 37°C were unable to oxidize arsenite when shaken with it at 25°C, and conversely, cells grown at 25°C were unable to oxidize arsenite when shaken with it at 37°C.

Summary of differential features.—The main differential features between the five types are tabulated in Table 5. Included in this are the corresponding features of *B. arsenoxydans*, as given by Green (1918*a*). The resemblance between it and our type III is evident.

TABLE 5

MAIN DIFFERENTIAL FEATURES OF ARSENITE-OXIDIZING BACTERIA

Criterion	Queensland Bacteria					*B.a.**
	I	II	III	IV	V	
gella polar	+	+	−	+	+	−
agella peritrichial	−	−	+	−	−	+
Colonies semi-fluid	+		−	−	−	−
Colonies very wrinkled	−	−	−	+	−	−
Pigment fluorescent, greenish yellow		+	−		−	−
Pigment brownish yellow, water-insoluble	−	−	−	+	−	−
Will grow at 3°C	−	+	−	−	−	0
Will grow at 44°C	−	−	−	+	−	0
Obligate aerobe	+	−	+	+	+	+
Facultative anaerobe	−	+	−	−	−	−
Produces acid from saccharides	+	+	−	+	−	−
Liquefies gelatine and coagulated serum	−		−	+	−	−
Breaks down nitrate to N_2	+	−	−	+	+	+
Breaks down nitrate to NH_3	−	+	+	−	−	−
Utilizes, as sole C source, starch and glycogen	−	−	−	+	−	0
Utilizes, as sole C source, maltose, raffinose, dextrin, and inulin, but not hexose	−	−	−	−	+	0
Utilizes, as sole C source, butyric acid	−	+	+	−	−	0
Utilizes, as sole C source, *p*-cresol	−	+	−	−	−	0
Unable to utilize, as sole C source, any saccharide	−	−	+	−	−	0
Facultative chemosynthetic autotroph	+	+	−	−	−	0

**B.a.* = *B. arsenoxydans* Green 1918; 0 = information not available.

(iii) *Taxonomy*

All the strains clearly fall into the class Schizomycetes Nägeli 1851, order Eubacteriales Buchanan 1917, sub-order Eubacteriineae Breed, Murray, and Hitchens 1944; all except strain 13 clearly fall into the family Pseudomonadaceae Winslow *et al.* 1917, and the tribe Pseudomonadeae Kluyver and van Niel 1936. Within this tribe, all the remainder except strain 16 fall into the genus *Pseudomonas* Migula 1894. Strain 16, because of its yellow, water-insoluble pigment and its proteolytic properties, is better placed in the genus *Xanthomonas* Dowson 1939. Strain 13 cannot be placed in the family Pseudomonadaceae as at present defined, because it possesses peritrichial flagella; it appears to fit best into the family Achromobacteriaceae Breed 1945, and the genus *Achromobacter* Bergey *et al.* 1923.

Study of Green's description of *B. arsenoxydans* suggests that, if it were available for study today, it too would be placed in the genus *Achromobacter*; Green had great difficulty in determining the type of flagellation, as we did with strain 13, but he finally decided that it was peritrichous. Strain 13 resembles Green's lost microorganism in many other ways (Table 5), but its identity with *B. arsenoxydans* Green 1918 can never be proved by direct comparison.

Bergey's Manual does not refer to any arsenite-oxidizing bacteria. Indeed, it is unlikely that any of the species listed there has ever been examined for capacity to oxidize arsenite, except by Green and Kestell (1918); they found that, although strains of bacteria now referred to as *Alcaligenes faecalis, Pseudomonas aeruginosa, Ps. fluorescens,* and *Ps. putida* could be accustomed to grow in the presence of 0·1M or even 0·15M arsenite, oxidation of arsenite never occurred. Even if arsenite oxidation is disregarded, none of our 15 strains can be fully identified with any of the 148 species of *Pseudomonas,* the 47 species of *Xanthomonas,* or the 12 species of *Achromobacter,* described in the 6th Edition. Type I resembles to some extent *Ps. ambigua, Ps. sinuosa,* and *Ps. cruciviae*; but, *inter alia,* it has unique undescribed colonial characteristics; type II resembles in some respects *Ps. putida,* but does not grow at 37°C, and does not produce an odour of trimethylamine; type III does not resemble any listed species of *Achromobacter,* or the closely related *Alcaligenes,* but we believe it to be closely related to, and possibly identical with *B. arsenoxydans* Green 1918; type IV does not resemble any listed species of *Xanthomonas*; and although type V resembles in many ways *Ps. sinuosa* (Wright) Chester 1901, the description given is not complete enough to justify identification.

It is proposed to regard these provisional types, possibly with the exception of type III, as new species of the appropriate genera, and to allot them the specific names "*arsenoxydans*" hyphenated to Latin cardinal numbers, because the ordinals have already been used as specific epithets with other genera (e.g. *Vibrio prima, Clostridium tertium*). The resulting compound epithets are relatively short, and are to that extent preferable to descriptive Latin epithets hyphenated to the already long "*arsenoxydans*"; furthermore, if it were later considered necessary to reduce the specific epithets to a single word, with "arsenoxydans" as a varietal epithet, the cardinal numbers could be retained as the specific epithets, and regarded as indeclinable particles in apposition with the generic epithets: if bacterial nomenclature is to be restricted to the Classical languages, some grammatical liberties may have to be tolerated. The proposed nomenclature is therefore as follows:

Type I : *Ps. arsenoxydans-unus* sp. nov.
Type II : *Ps. arsenoxydans-duo* sp. nov.
Type III : *Achr. arsenoxydans-tres* sp. nov.
Type IV : *Xanth. arsenoxydans-quattuor* sp. nov.
Type V : *Ps. arsenoxydans-quinque* sp. nov.

(*d*) *Some Consequences of Arsenite Oxidation During Growth*

(i) *Concomitant Decrease of pH*.—Because arsenic acid dissociates much more than does arsenious acid, the pH falls considerably as oxidation proceeds, even in a moderately well-buffered medium like KA3. This leads to an inverse relationship between the degree of oxidation and the pH, which is illustrated, with *Ps. arsenoxydans-duo* as an example, in Figure 3; in this instance the pH decreased 1·3 pH units, whereas in Koser medium without arsenite the change in pH was insignificant. Other species behaved similarly, although differences occurred in the extent of pH decrease, probably dependent upon the capacity of the particular strain to give rise to alkali from citrate.

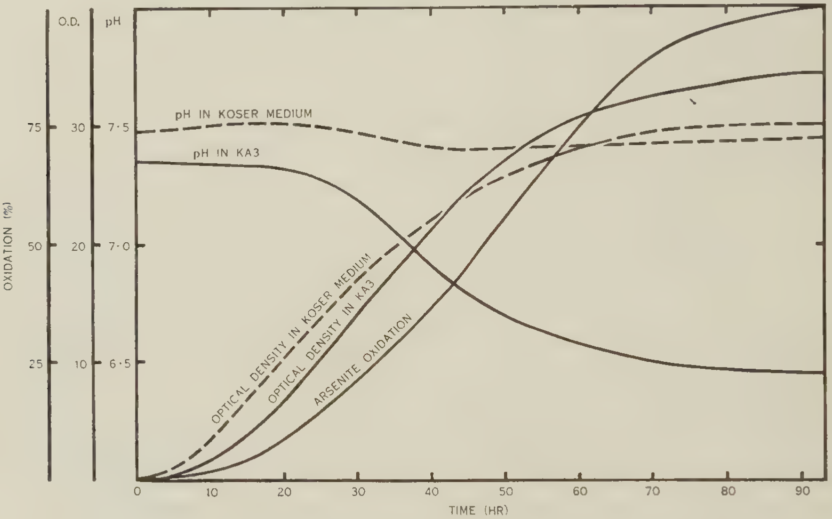

Fig. 3.—Inverse relationship between the percentage oxidation and the pH of *Ps. arsenoxydans-duo*, strain 5, grown in KA3 at 25°C.

(ii) *pH Optimum for Growth in KA3*.—*Ps. arsenoxydans-quinque* was sown into KA3 adjusted to the following pH values: 4·2, 5·1, 6·1, 7·2, 7·7, 9·4, 9·8, and 10·7. After 143 hr, growth and oxidation had reached virtual completion at all pH values between 6·1 and 9·4, but failed entirely at pH 9·8 and 10·7; at pH 5·1 oxidation had reached 26·9 per cent., but at pH 4·1 it had reached only 4·6 per cent. (Fig. 4).

This experiment revealed a wide pH tolerance for growth in an arsenical medium. The very sharp difference between the behaviour at pH 9·8, at which growth failed, and at pH 9·3, at which it was almost maximum, is to be explained by the fall in pH which accompanies oxidation; in this instance, the pH had fallen in 143 hr to 6·8, and it therefore appears that if the pH is not too alkaline to permit growth to start, the concomitant oxidation of arsenite progressively lowers the pH to more favourable levels for growth. When the initial pH is on the acid side of the optimum, the opposite effect must come into play. It happens that in arsenical dipping fluids the initial pH is high—of the order of 9·5—but not beyond the limit at which growth can begin.

(iii) *Progress of Oxidation in a "Model Dip."*—This is further illustrated in the following experiment. Commercial dip concentrate was diluted with tap water to prepare the standard dipping fluid, containing 0·2 per cent. As_2O_3, corresponding to approximately 0·02M arsenite, with pH = 9·55. In this was incorporated a filtered boiled decoction of bovine faeces, corresponding to 4·5 g dried faeces per l. It was placed in an earthenware jar, the depth of fluid being 16·5 cm, and 10 ml of a culture of *Ps. arsenoxydans-quinque* were added. The covered jar was left at room temperature (20°-10°C). The progress of oxidation, and the pH, are shown in Figure 5.

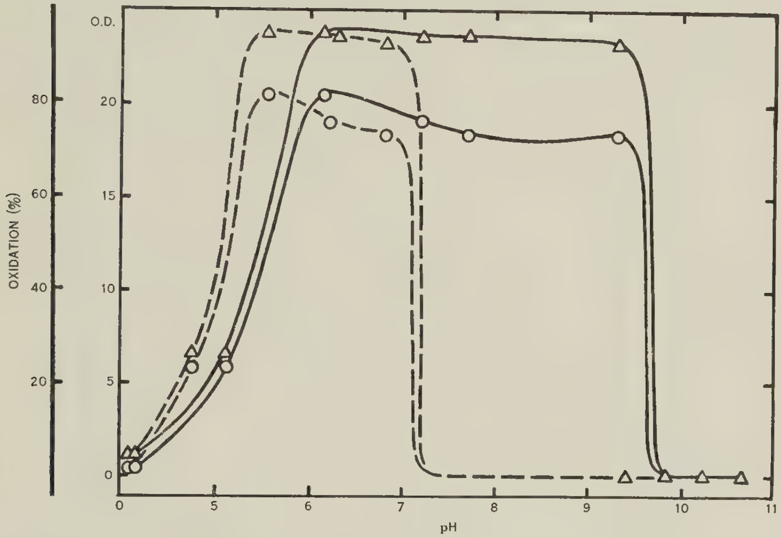

Fig. 4.—Influence of initial pH of KA3 upon growth and percentage oxidation by *Ps. arsenoxydans-quinque*, strain 17, at 25°C, after 143 hr. In the curves with continuous lines, the values are related to the original pH's; in the curves with broken lines, they are related to final pH's after 143 hr.

It is seen that oxidation began quite soon, and proceeded rapidly to reach a maximum level of more than 80 per cent. after about 55 days. During this period the pH fell inversely, and reached less than pH 4, doubtless because the buffering by faecal decoction was very much weaker than in KA3; probably the low pH prevented further growth and oxidation.

Records of samples of spontaneously oxidized dipping fluids received from the field suggest that in practice the pH does not often fall below pH 6 (Hitchcock, personal communication); this is partly because oxidation usually does not proceed so far under field conditions, where the activity of arsenate-reducing bacteria may also operate simultaneously (Green 1918*b*), and partly because accumulation of phosphate from excreta gradually improves the buffering properties.

(iv) *Influence of Arsenite Concentration.*—*Ps. arsenoxydans-quinque* was sown into Koser medium at pH 7·6, containing 0·02, 0·04, 0·06, 0·08, and 0·10M arsenite. After 5 days at 25°C, growth had occurred equally well in the presence of 0·02 and 0·04M arsenite, but it was slightly inhibited at 0·06M, considerably inhibited at 0·08M, and almost completely inhibited at 1·0M. This is illustrated in Figure 6, which shows that, up to 0·06M arsenite, there was a positive correlation between growth, progress of oxidation, and pH; beyond 0·06M, the inhibitory effect of arsenite became apparent.

As incubation proceeded, growth improved even at the higher concentrations of arsenite, and by the twelfth day it was quite strong even in the presence of 0·10M arsenite.

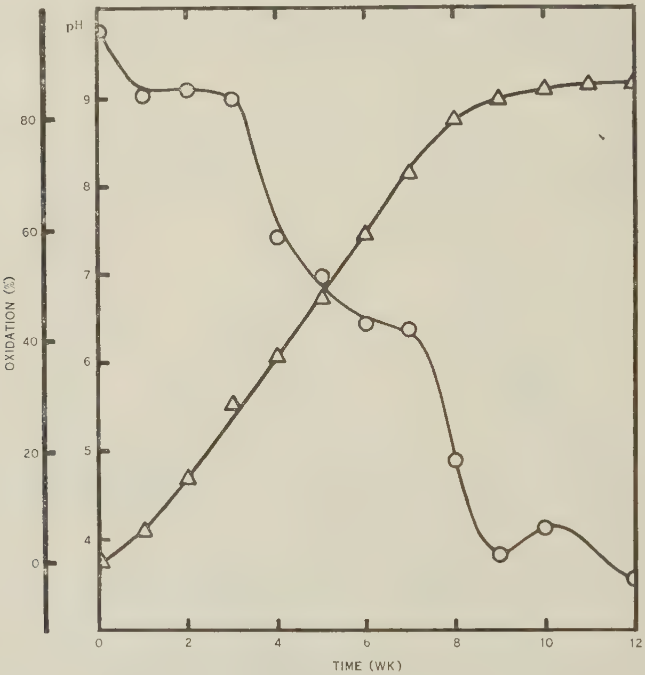

Fig. 5.—Progress of oxidation, and concomitant decrease of pH, in commercial dipping fluid enriched with faecal extract, sown with *Ps. arsenoxydans-quinque*, strain 17, and incubated at room temperature.

IV. Discussion

The development of enzyme systems for the oxidation of arsenite to the less toxic arsenate suggests itself as one mechanism for protecting vulnerable thiol groups from inactivation by arsenite. However, that this property is not essential to high tolerance towards arsenite is clear from the findings of Green and Kestell (1918) that certain arsenite-resisting fluorescent and non-fluorescent pseudomonads isolated from cattle-dipping fluids could be trained to tolerate 0·1M arsenite or more, without showing any evidence of arsenite oxidation. Nevertheless, it seems possible that, with the group of arsenite-oxidizing bacteria described in this paper, capacity to oxidize arsenite may favour survival in an arsenical dip; we have observed that when cells of *Ps. arsenoxydans-unus* were held under anaerobic conditions in KA3, so that growth and oxidation were inhibited, the viable count began to fall, whereas, on admitting air, growth and arsenite oxidation followed a normal course. Further experiments of this sort, particularly with the facultatively anaerobic *P. arsenoxydans-duo*, are desirable to settle this point. A definite biological advantage of arsenite oxidation to these bacteria is the reduction of pH to values nearer the optimum for their growth, as the pH of freshly prepared dipping fluids is near the high limit of their pH tolerance. Calculation from the heats of formation of the acids in dilute solution indicates that oxidation of arsenite to arsenate could provide about 77 kcal per mole, which is comparable with the energy of anaerobic fermentation of glucose, for example; however, there was no evidence in the limited experiments that these organisms are able to utilize the energy of arsenite oxidation for growth purposes.

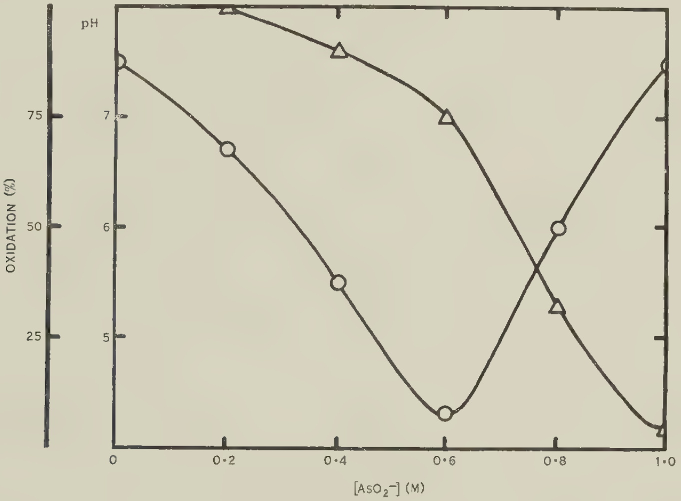

Fig. 6.—Influence of concentration of arsenite upon growth, percentage oxidation, and decrease of pH by *Ps. arsenoxydans-quinque*, strain 17, after 5 days at 25°C. The inhibition by $[AsO_2^-] > 0\cdot06$M, apparent after 5 days was eventually overcome, and quite good growth even in 0·10M arsenite had occurred after 12 days.

The ultimate origin of arsenite-oxidizing strains, whether from soil, water, or faeces, cannot be stated with certainty. In experiments not reported here, attempts were made to accustom a dozen assorted strains of fluorescent pseudomonads, of South Australian origin, which had been isolated from soil, water, and pathological conditions; in no instance could they be trained to tolerate more than 0·01M arsenite in Koser medium, and none was able to oxidize it. Furthermore, we were unable to demonstrate arsenite-oxidizing bacteria in the faeces of horses and cows at Melbourne, when small amounts were sown into KA3 medium. However, when 40 g fresh bovine faeces collected from the cement floor of stalls was added to 20 l. of dipping fluid made with tap water from commercial concentrate, and left at room temperature, oxidation was detected within a week; it eventually reached 83 per cent., and an arsenite-oxidizing pseudomonad, different from those described from Queensland material, was isolated. As the animal which was the source of the faeces was of local origin, and as the nearest arsenical cattle dips are over 600 miles away, it is unlikely that the pseudomonad isolated had had previous experience of arsenite; it could have been present in the faeces, on the concrete floor, or in the tap water, but it presumably had the potentiality to develop not only arsenite tolerance but an arsenite-oxidizing enzyme system. Green and Kestell (1918) found that arsenite-tolerant bacteria, without oxidizing power, were common in cattle faeces, but did not occur in soil; the genus *Bacillus,* and the "colon-typhoid group" had a low tolerance, but pseudomonads and *Alcaligenes faecalis* could be trained to withstand high concentrations.

Some discussion is called for on the classification of our strains. Fourteen of the strains are undoubtedly pseudomonads; 13 quite clearly belong to the genus *Pseudomonas,* but one (strain 16) is so different in colonial characteristics, pigment formation, and proteolytic properties that no objection can be raised against regarding it as a separate species, and a different genus. We consider it best placed in the genus *Xanthomonas,* and not in the genus *Flavobacterium,* because it has polar flagella. We have classified strain 13 in the genus *Achromobacter* because it is peritrichially flagellated, is inert towards carbohydrates, alcohols, etc., and does not produce pigment; but a case could be made for regarding it as an *Alcaligenes* with poor alkalinizing properties; indeed, Green (1918*a*) was inclined to classify his closely similar bacillus in this way, but was dissuaded because of the absence of alkali production in milk, and he was uncertain of the flagellation. At the present stage of taxonomy, the type of flagellation is accorded very great weight. Admittedly, the recent demonstration by Leifson and Hugh (1953) that the flagellar arrangement of *Aeromonas,* of *Xanthomonas,* of *Flavobacterium,* and of a *Vibrio* could undergo variation of several kinds, and that the formation of chains or filaments by polar flagellates could give the appearance of peritrichous flagellation, has thrown doubt on the validity of this criterion. The main matter on which some difference of opinion may exist is the differentiation of the three species allotted to the genus *Pseudomonas.* Type II, which is fluorescent and relatively cryophilic, can be clearly separated. Types I and V are rather similar in most cultural characteristics except colony shape on solid media. As pseudomonads are prone to develop mutants, this feature alone might not be sufficient for specific rank, but the very profound differences in dissimilative ability, and in the capacity to utilize carbon sources, justify their separation in our view.

Finally, some comment is appropriate on our finding that several species of bacteria are capable of oxidizing arsenite. On reflection, this appears no more surprising than that diverse species, with otherwise distinct characteristics, should be able to oxidize sulphur or hydrogen. It is possible that if Green had extended his investigation beyond the dip at Onderstepoort he might have found other oxidizing species.

V. Acknowledgments

Thanks are due to the following colleagues: Miss C. E. Eales, who assisted in the early stages of the investigation; Mr. A. T. Dann, who kindly purified, synthesized, or provided many test substances for the C-source utilization tests; Mr. E. Wold, who showed skill and patience in photographing preparations of flagellated bacteria; the late Mr. L. F. Hitchcock, Division of Entomology, C.S.I.R.O., who forwarded most of the samples and gave useful advice on the problem; and Dr. A. J. Hodge, Division of Industrial Chemistry, C.S.I.R.O., who prepared the electron micrographs. Mr. L. G. Newton, Queensland Department of Agriculture and Stock, forwarded samples 16 and 17, and provided much useful information, and Mr. E. French, Walter and Eliza Hall Institute for Medical Research, Royal Melbourne Hospital, kindly provided a collection of strains of fluorescent pseudomonads, and tendered valued advice.

It is especially desired to record our indebtedness to Mr. N. E. Southern for conscientious and skilled technical assistance.

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Explanation of Plates 1 and 2

Plate 1

Figs. 1-5.—*Ps. arsenoxydans-unus*: Fig. 1.—Colonies on McConkey agar (MCA) 10 days, × 2·5. Fig. 2.—24-hr Koser, electron micrograph, × 5600. Fig. 3.—Colonies on MCA, 10 days, transmitted light, × 2·5. Fig. 4.—Petri dish confluent growth on

MCA, 14 days, showing colonies flowing slowly down sides of dish, × 0·5. Fig. 5.—Smear, stained dil. basic fuchsin, PA, 2 days, × 2000.

Figs. 6-10.—*Ps. arsenoxydans-duo*: Fig. 6.—Colonies on PA, 4 days, × 10. Fig. 7.—Colonies on PA, 14 days, × 10. Fig. 8.—Smear, stained dil. basic fuchsin, PA, 2 days, × 2000. Fig. 9.—Smear, stained dil. basic fuchsin, Koser, 24 hr, × 2000. Fig. 10.—Smear, Leifson's flagella strain, Witte peptone veal infusion (WPVI), 2 days, × 2000.

Plate 2

Figs. 1-4.—*Achr. arsenoxydans-tres*: Fig. 1.—Colonies on PA, 4 days, × 10. Fig. 2.—Smear, stained dil. basic fuchsin, PA, 2 days, × 2000. Fig. 3.—Smear, stained dil. basic fuchsin, KA3, 2 days, × 2000. Fig. 4.—Smear, Leifson's flagella stain, WPVI, 2 days, × 2000.

Figs. 5-9.—*Xanth. arsenoxydans-quattuor*: Fig. 5.—Colony on PA, 3 days, × 10. Fig. 6.—Confluent growth on PA, 3 days, × 10. Figs. 7 and 9.—Smear, Leifson's flagella stain, WPVI, 2 days, × 2000. Fig. 8.—Smear, stained dil. basic fuchsin, PA, 2 days, × 2000.

Figs. 10-13.—*Ps. arsenoxydans-quinque*: Fig. 10.—Colonies on PA, 3 days, × 10. Fig. 11.—Smear, stained dil. basic fuchsin, WPVI, 2 days, × 2000. Fig. 12.—Smear, stained dil. basic fuchsin, PA, 2 days, × 2000. Fig. 13.—Smear, Leifson's flagella stain, WPVI, 2 days, × 2000.

Appendix I

MONOGRAPHS OF THE PROPOSED NEW SPECIES[*]

The techniques used for certain of the tests were as follows:

(i) *Nitrate Reduction.*—The medium of Spray (1936) with the diphenylamine test for nitrate, the α-naphthylamine-sulphonic acid test for nitrite, and gas bubbles as evidence of reduction to N_2, NO_2, or both.

(ii) *Indole Production.*—Bacto-Tryptone peptone water, with the vanillin reagent.

(iii) *H_2S Production.*—Bacto lead acetate agar.

(iv) *Arsenite Oxidation.*—KA3 medium.

1. *Pseudomonas arsenoxydans-unus* sp. nov.

Rods: 0·9-1·0 by 1·6 by 2·3 μ (measured from electron micrographs of cells grown in Koser's citrate medium); 0·4-0·6 by 1-2 μ (measured from photomicrographs of stained smears of cells grown on potato extract peptone agar). Tendency to form radiating clumps or rosettes (Plate 1, Fig. 5). Motile; single polar flagellum up to 7 μ long, with 2½ undulations (Plate 1, Fig. 2). Gram-positive. Spores not formed.

Pigment: None.

Gelatin: Not liquefied.

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[*] Dried subcultures of the type strains have been submitted to the National Collection of Type Cultures, London.

Agar: Circular, domed, glistening, creamy colonies which may later develop greyish flecks or streaks; very soft and semi-fluid. Readily coalesce when contiguous (Plate 1, Fig. 1). By transmitted light, translucent with denser light brownish centres (Plate 1, Fig. 3). Colonies may eventually reach 2 cm in diameter; they readily flow under the influence of gravity, and may drip to the lower lid of an inverted petri-dish culture (Plate 1, Fig. 4).

When grown in presence of arabinose, xylose, adonitol, or erythritol, variants may develop which form small, whitish, opaque, circular, entire, raised colonies with a crateriform depression.

Broth: Turbid growth with formation of slight pellicle; later a tenacious mucinous deposit.

Litmus milk: Slowly becomes slightly alkaline, with decolorization in the depth.

Inspissated serum: Not liquefied.

Potato slope: Colourless, confluent growth.

Nitrate: Broken down to N_2.

Catalase: Positive.

Indole: Not formed.

Hippurate: Split.

Hydrogen sulphide: Small amount formed.

Oxygen requirements: Strongly aerobic. Does not grow anaerobically in meat infusion peptone broth. Very slight growth anaerobically on potato infusion agar or on blood agar. Grows in the depths of nitrate agar, or along the needle track in gelatine stabs, but only on the surface of glucose agar shakes.

Fermentations: Type of attack is oxidative. Gas not produced.

(*a*) In peptone water, acid as follows: arabinose, xylose, rhamnose, glucose, fructose, erythritol, and adonitol. *Not* with mannose, galactose, saccharose, maltose, lactose, trehalose, raffinose, starch, inulin, dextrin, glycogen, methanol, ethanol, glycerol, mannitol, dulcitol, sorbitol, salicin, aesculin, amygdalin, or inositol.

(*b*) With cells harvested from potato extract peptone agar, acid as follows: arabinose and xylose (weak); glucose (strong), fructose (weak). *Not* with any of other substances listed above.

Carbon sources utilized: All those listed above, *except* lactose, raffinose, starch, glycogen, salicin. In addition, utilizes following acids: formic, acetic, lactic, oxalic, mucic, tartaric, ascorbic, glycollic, succinic, fumaric, malic, aconitic, pyruvic, mandelic, adenylic, *p*-hydroxybenzoic, *p*-hydroxyphenylacetic, *p*-aminobenzoic, pimelic, phthalic, β-glycerophosphoric, uric; *but not* butyric, benzoic, *o*-aminobenzoic, salicylic, phenylpropionic, or α-naphthylacetic acid. Also utilizes phenol, aniline, pyridine, α-picoline, *s*-collidine, diethyleneglycol, *cyclo*hexanol, methyl-*cyclo*hexanone, glucose-1-phosphate, and fructose-1, 6-diphosphate; *but not* *p*-cresol.

Temperature requirements: No appreciable growth in 4 wk at 3°C; grows at 37°C but not at 44°C. Optimum near 25°C.

Arsenite oxidation: In Koser's citrate medium containing 0·02M arsenite, complete oxidation in 11 days at 25°C; less active at 37°C.

Source: Ten arsenical cattle-dipping fluids from southern Queensland. "Strain 1" is regarded as the type strain.

2. *Pseudomonas arsenoxydans-duo* sp. nov.

Rods: Rather sharply divided into two sizes of cells (Plate 1, Fig. 8): smaller 0·5 by 1 μ, larger 1 by 6 μ (cells grown on potato extract peptone agar). Cells grown in Koser medium not so thick (0·7 μ) (Plate 1, Fig. 9). Motile; flagella 5-6 μ long with 2½ undulations; single or in tufts up to four (Plate 1, Fig. 10). Gram-negative. Spores not formed.

Pigment: Greenish yellow fluorescent pigment.

Gelatin: Not liquefied. Slightly arborescent growth along the stab.

Agar: Large creamy-white, circular, entire, domed colonies (Plate 1, Fig. 6); later develop flattened margin (Plate 1, Fig. 7).

Broth: Heavy turbid growth without pellicle; later a heavy deposit which readily resuspends. Greenish yellow fluorescence.

Litmus milk: Slowly becomes slightly alkaline, with decolorization in the depths.

Inspissated serum: Not digested.

Potato slope: Confluent growth, very slight brown pigmentation.

Nitrate: No gas; reduction to nitrite, and later to NH_3.

Catalase: Positive.

Indole: Not formed.

Hippurate: Split.

Hydrogen sulphide: Small amounts formed.

Oxygen requirements: Grows only slightly under anaerobic conditions on surface of potato extract, peptone agar, blood agar, and McConkey agar, but grows well anaerobically in nutrient broth, in Koser medium, and in KA3.

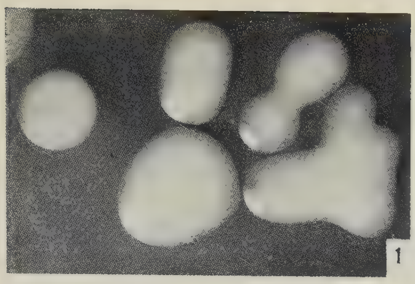
Fermentations: Type of attack is oxidative. Gas not produced.

(*a*) In peptone water, acid as follows: xylose, arabinose, glucose, and fructose. *Not* with rhamnose, mannose, galactose, with any di-, tri-, or polysaccharides, with any mono-, di-, tri-, tetra-, penta-, or hexahydric alcohols, with glucosides, or with inositol.

(*b*) With cells harvested from potato extract peptone agar, acid as follows: arabinose, xylose, *but not* rhamnose; glucose, mannose, galactose, *but not* fructose; raffinose and polysaccharides not attacked; of the disaccharides, only maltose is attacked; of the alcohols, only ethanol and dulcitol are attacked; aesculin and amygdalin are attacked, *but not* salicin; inositol *not* attacked.

Carbon sources utilized: Arabinose, xylose, glucose, fructose, galactose, maltose, inulin, dextrin: methanol, ethanol, glycerol; amygdalin; glucose-1-phosphate; fructose-1, 6-diphosphate. Acids utilized: formic, acetic, lactic, oxalic, mucic, tartaric, ascorbic, glycollic, succinic, fumaric, malic, aconitic, pyruvic, mandelic, adenylic, butyric, *p*-hydroxyphenylacetic, benzoic, *p*-hydroxybenzoic, pimelic, phthalic, β-glycerophosphoric, phenylpropionic, uric, and a-naphthylacetic; *but not* *p*-aminobenzoic, *o*-aminobenzoic, or salicylic acid.

BACTERIAL OXIDATION OF ARSENITE. I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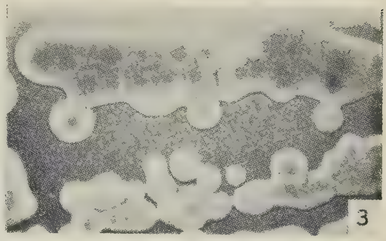
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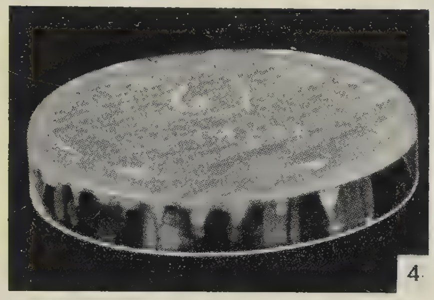
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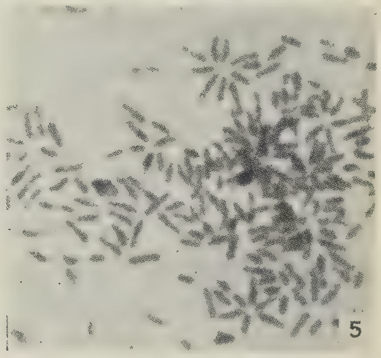
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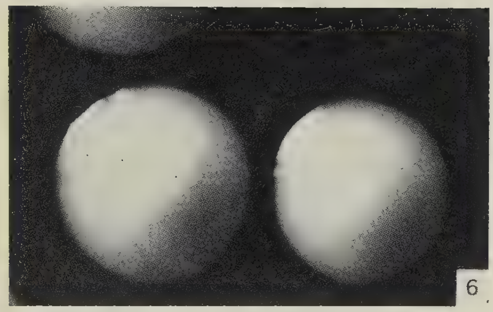
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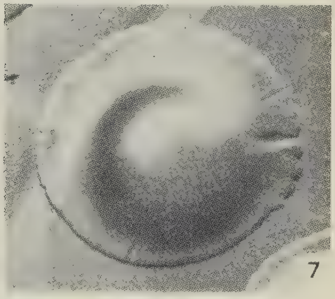
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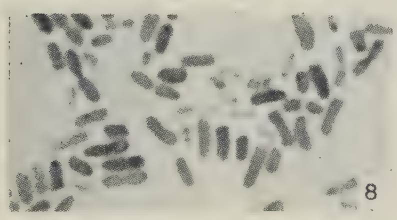
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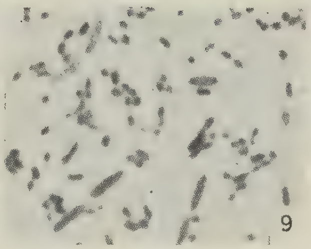
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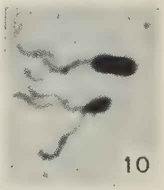
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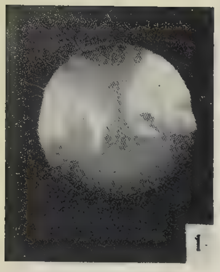
BACTERIAL OXIDATION OF ARSENITE. I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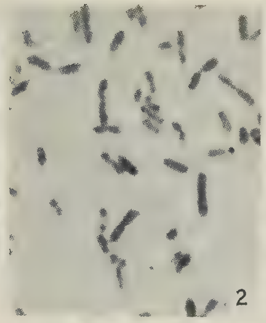
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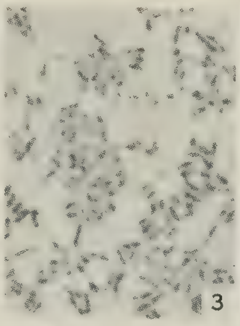
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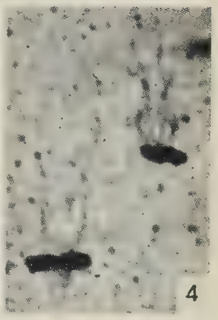
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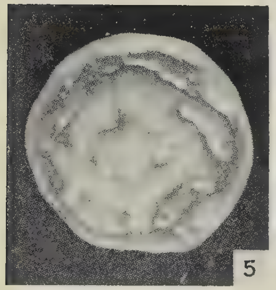
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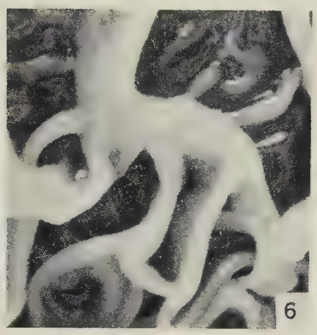
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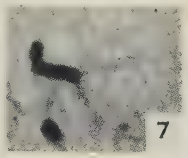
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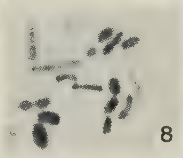
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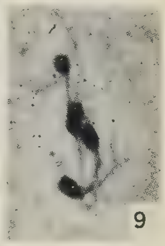
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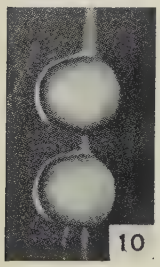
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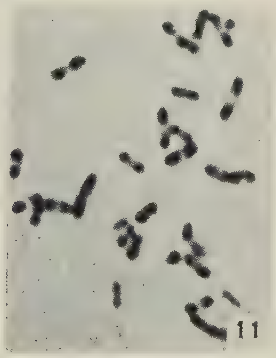
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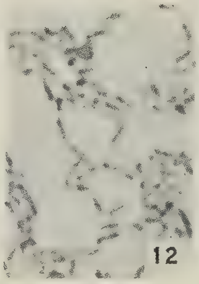
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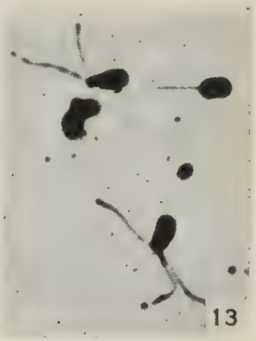
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Also utilizes phenol, aniline, *p*-cresol, pyridine, α-picoline, *s*-collidine (one strain), diethyleneglycol, methyl-*cyclo*hexanone; *but not cyclo*hexanol.

Temperature requirements: Grows well on surface of potato peptone agar and in infusion peptone broth at 3°C; also at 25°C; but not at 37°C or higher. Optimum probably near 25°C.

Arsenite oxidation: In KA3 complete oxidation of 0·02M arsenite in 7 days at 25°C; does not grow at 37°C.

Source: Two arsenical cattle-dipping fluids from near south coast of Queensland. "Strain 5" is regarded as the type strain.

3. *Achromobacter arsenoxydans-tres* sp. nov.

Rods: 0·5 by 0·6 μ to 0·7 by 3 μ (cells grown on potato extract peptone agar) (Plate 2, Fig. 2); 0·35 by 0·5 μ to 0·45 by 2 μ (cells grown in KA3 medium) (Plate 2, Fig. 3). Motile; flagella 5-6 μ long, with 2½ undulations; 8-14 flagella, peritrichial (Plate 2, Fig. 4). Gram-negative. Spores not formed.

Pigment: Not formed.

Gelatin: Not liquefied.

Agar: Circular, entire, domed creamy colonies (Plate 2, Fig. 5); later a flat periphery develops.

Broth: Heavy turbid growth without pellicle; later a heavy deposit which readily resuspends.

Litmus milk: Slowly becomes slightly alkaline, with decolorization in the depths.

Inspissated serum: Not liquefied.

Potato slope: Colourless, confluent growth.

Nitrate: Gas not formed; reduction to nitrite, and later to NH_3.

Catalase: Positive.

Indole: Not formed.

Hippurate: Split.

Hydrogen sulphide: Small amount formed:

Oxygen requirements: Strongly aerobic; under anaerobic conditions, very slight growth on blood agar, but no growth on potato peptone agar, McConkey agar, in nutrient broth, in Koser medium, or in KA3. Grows in depths of nitrate media, but only on surface of glucose agar shakes.

Fermentations: Acid is not produced from pentoses, hexoses, saccharides, alcohols (mono-, di-, tri-, tetra-, or hexahydric), glucosides, or inositol, either by cultures in peptone water or by thick suspensions of cells harvested from potato extract peptone agar.

Carbon sources utilized: Of the same large range of substances, only methanol, ethanol, inulin, and amygdalin are able to support growth. Fructose-1, 6-diphosphate, *but not* glucose-1-phosphate, is also utilized. On the other hand, it can utilize a large variety of organic acids, namely formic, acetic, lactic, oxalic, tartaric, glycollic, succinic, fumaric, malic, aconitic, pyruvic, adenylic, butyric, *p*-hydroxyphenylacetic, *p*-aminobenzoic, pimelic, phthalic, and β-glycerophosphoric acid; *but not* mucic, *o*-aminobenzoic, ascorbic, salicylic, phenylpropionic, uric, or α-naphthylacetic acids. It utilizes aniline, pyridine, α-picoline, *s*-collidine, diethyleneglycol, *cyclo*hexanol, and methyl-*cyclo*hexanone, *but not* phenol or *p*-cresol.

Temperature requirements: It does not grow appreciably at 3°C, but grows at 25°C and 37°C. The optimum is around 25°C.

Arsenite oxidation: In KA3, complete oxidation of 0·02M arsenite in 20 days at 25°C; slightly more active at 37°C.

Source: An arsenical cattle-dipping fluid from Gladstone, Qld. "Strain 13" is the type strain.

4. *Xanthomonas arsenoxydans-quattuor* sp. nov.

Rods: 0·3 by 1 μ to 1 by 3·5 μ (cells grown on potato extract peptone agar) (Plate 2, Fig. 8). In infusion peptone broth, forms chains and long, sinuous filaments. Motile; up to six polar flagella 5-6 μ long, with 2½ undulations (Plate 2, Figs. 7 and 9). Gram-negative. Spores not formed.

Pigment: Bright yellow or brownish yellow; water-insoluble.

Gelatin: Liquefied.

Agar: Pale yellow colonies which become tough and very wrinkled on continued incubation (Plate 2, Fig. 5); confluent growth develops great irregular folds (Plate 2, Fig. 6).

Broth: Turbid growth, slight pellicle which may sink; mucinous deposit later.

Litmus milk: Slowly becomes slightly alkaline, with decolorization in the depths.

Inspissated serum: Liquefied.

Potato slope: Thick, firm, tough, gelatinous, glistening, strongly wrinkled, yellowish brown.

Nitrate: Reduced to N_2.

Catalase: Positive.

Indole: Not formed.

Hippurate: Not split.

Hydrogen sulphide: Small amount formed.

Oxygen requirements: Strongly aerobic; under anaerobic conditions, no growth in any medium except in depths of nitrate agar, or along needle track in gelatine stabs; grows only on the surface of glucose agar shakes.

Fermentations: Type of attack oxidative. Gas not produced. With cells harvested from potato extract peptone agar, acid as follows: arabinose and xylose (weak); glucose, mannose and galactose (strong), fructose (weak); aesculin (weak).

Carbon sources utilized: Xylose, glucose, fructose, galactose, maltose, starch, dextrin, glycogen, methanol, ethanol, glycerol, mannitol, and amygdalin; *but not* arabinose, rhamnose, mannose, lactose, saccharose, trehalose, inulin, erythritol, adonitol, dulcitol, sorbitol, salicin, aesculin, or inositol. In addition, utilizes following acids: formic, acetic, lactic, oxalic, tartaric, ascorbic, glycollic, succinic, fumaric, malic, aconitic, pyruvic, adenylic, *p*-hydroxyphenylacetic, phthalic, β-glycerophosphoric, uric, α-naphthylacetic; *but not* mucic, butyric, benzoic, *p*-hydroxybenzoic, *p*-aminobenzoic, *o*-aminobenzoic, or salicylic acid. Also utilizes phenol, aniline, pyridine, α-picoline, diethyleneglycol, methyl-*cyclo*hexanone, glucose-1-phosphate and fructose-1,6-diphosphate; *but not* *p*-cresol, *s*-collidine, or *cyclo*hexanol.

Temperature requirements: No appreciable growth at 4°C; grows at 25°C, 37°C, and even 44°C. Optimum near 25°C.

Arsenite oxidation: In KA3, complete oxidation of 0·02M arsenite in 12 days at 25°C, but cultures are unable to oxidize arsenite at 37°C or 44°C.

Source: An arsenical cattle-dipping fluid from Stuart's Creek, Qld. "Strain 16" is the type strain.

5. *Pseudomonos arsenoxydans-quinque* sp. nov.

This species (strain 17) was chosen for study of enzymic mechanism of arsenite oxidation (Turner 1949; Turner and Legge 1954; Legge and Turner 1954; Legge 1954).

Rods: 0·45-0·50 by 1-2·3 μ (on potato extract agar) (Plate 2, Fig. 11); 0·5-0·8 by 1·5-3·5 μ (in meat infusion peptone broth) (Plate 2, Fig. 12). Cells stain bipolarly. Motile; one to three polar flagella 5-6 μ long, with 2½ undulations (Plate 2, Fig. 13). Gram-negative. Spores not formed.

Pigment: Virtually none; slight amounts of yellowish pigment associated with the deposit of cells in old cultures in Koser or KA3 media.

Gelatin: Slight softening after long incubation, but no liquefaction.

Agar: Small circular domed creamy whitish colonies with entire edge (Plate 2, Fig. 10).

Broth: Turbid growth, relatively slow to settle, thick mucinous deposit.

Litmus milk: Slowly becomes slightly alkaline, with decolorization in the depths.

Inspissated serum: Not liquefied.

Potato slope: Colourless confluent growth.

Nitrate: Reduced to N_2.

Catalase: Positive.

Indole: Not formed.

Hippurate: Not split.

Hydrogen sulphide: Small amount formed.

Oxygen requirements: Strongly aerobic; under anaerobic conditions, no growth in any medium. Grows in depths of nitrate agar, or along the needle track in gelatin stabs, but only on the surface of glucose agar shakes.

Fermentations: With cells from potato extract peptone agar, acid is not produced from any pentoses, hexoses, di-, tri-, and polysaccharides, alcohols, glucosides, or inositol.

Carbon sources utilized: Xylose, maltose, raffinose, inulin, dextrin, methanol, ethanol, glycerol, mannitol, amygdalin, glucose-1-phosphate, and fructose-1, 6-diphosphate; *but not* arabinose, rhamnose, any hexose, sucrose, lactose, starch, glycogen, erythritol, adonitol, dulcitol, sorbitol, inositol, salicin, or aesculin. In addition, utilizes following acids: formic, acetic, lactic, oxalic, mucic, tartaric, succinic, fumaric, malic, aconitic, pyruvic, adenylic, *p*-hydroxyphenylacetic, *p*-aminobenzoic, pimelic, phthalic, β-glycerophosphoric, uric, α-naphthylacetic;

but not ascorbic, butyric, benzoic, *o*-aminobenzoic, or *p*-aminobenzoic acid. Also utilizes phenol, aniline, pyridine, *a*-picoline, *s*-collidine, diethyleneglycol, methyl-*cyclo*hexanone, glucose-1-phosphate, and fructose-1, 6-diphosphate; *but not p*-cresol or *cyclo*hexanol.

Temperature requirements: Does not grow at 3°C or at 44°C, but grows at 25°C and 37°C; optimum near 25°C.

Arsenite oxidation: In KA3, complete oxidation of 0·03M arsenite in 6 days when grown at 25°C, but cultures are unable to oxidize arsenite in KA3 when grown at 37°C. Cells grown in KA3 at 25°C can oxidize arsenite at 37°C (Turner 1949).

Source: An arsenical cattle-dipping fluid from Richmond, Qld. "Strain 17" is the type strain.

BACTERIAL OXIDATION OF ARSENITE

II. THE ACTIVITY OF WASHED SUSPENSIONS

By A. W. TURNER[*] and J. W. LEGGE[†]

[*Manuscript received June 2, 1954*]

Summary

Maximum arsenite-oxidizing activity of a pseudomonad (*Ps. arsenoxydans-quinque*) isolated from "oxidized" arsenical cattle-dipping fluids was found in cells 3-4 days old, when cultivated in a synthetic medium containing arsenite. The system is adaptive, cells cultivated in the absence of arsenite showing no arsenite-oxidizing activity.

The oxidizing system in cells harvested at peak activity was optimally active in air at pH 6·4 and 40°C, when tested in several different buffers. It was inhibited by cyanide and azide. Light-sensitive CO inhibition was demonstrated 6 months after the strain was first isolated, but 22 months later, after much subculturing, this property had been virtually lost. Inhibition was also observed with fluoride and pyrophosphate. Certain dicarboxylic acids produced a temporary inhibition between pH 4 and 5. Iodoacetate, diethyldithiocarbamate, α,α'-dipyridyl, and urethane were without effect.

Cells were unable to oxidize arsenite in presence of air after treatment with toluene, acetone, or after being subjected to desiccation.

Since adapted cells can oxidize arsenite anaerobically in the presence of suitable acceptors, it is concluded that the complete system includes an arsenite dehydrogenase and cytochromes.

I. INTRODUCTION

In a previous communication a description was given of 15 strains of arsenite-oxidizing bacteria, comprising five types, isolated from "oxidized" cattle-dipping fluids in Queensland (Turner 1954). Since tervalent arsenite is known to inactivate widely distributed enzymes whose activity depends on $-SH$ groups (Stocken and Thompson 1949), study of the arsenite-oxidizing mechanism of these bacteria, most of which are pseudomonads, may not only be of theoretical biochemical interest, but may throw light on the mechanism by which other microorganisms develop resistance to arsenite and its derivatives.

This paper is concerned with the conditions for obtaining cells with maximum arsenite-oxidizing activity, and with some of the properties of the system in intact cells.

II. METHODS

The experiments were carried out with strain 17, which has been allotted to the species *Pseudomonas arsenoxydans-quinque* (Turner 1954); it grows well,

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and provides a high yield of very active cells. Usually these were grown in liquid cultures in KA3 medium (Turner 1954), i.e. Koser citrate medium containing 0·02M $NaAsO_2$. Centrifuged cells could be stored at 4°C for some weeks with very little loss of activity. For use, they were washed, usually once, in the original volume of 0·067M phosphate buffer (pH 6·4), and finally were resuspended in the appropriate buffer. The turbidity was measured in a photoelectric absorptiometer, and in most experiments the volume was adjusted with buffer to a cell concentration containing 0·13 mg dry weight per ml.

The velocity of arsenite oxidation was followed by means of iodometric titration; aeration was obtained by shaking the suspensions in rubber-stoppered 50-ml flasks with a Microid shaker, samples being withdrawn at intervals for titration. Arsenite oxidation was also followed by conventional manometric methods, and by dye reduction in Thunberg tubes.

Strain 17, which was isolated in December 1947, had been maintained by irregular subculturing in KA3 medium. Most of the work reported in the preliminary publication (Turner 1949), including the CO inhibitions, was carried out by June 1948. The investigation of soluble arsenite dehydrogenase was begun in 1949. By October 1949, it was found that cells were giving very low yields of arsenite dehydrogenase, and examination revealed two main colonial types in the culture, of which substrain *L*2 was selected as highly active for this purpose. In March 1950 when the work on CO inhibition was taken up again, it was found that the phenomenon could be obtained only very irregularly with this substrain, which appears to differ from the original isolation.

III. RESULTS

(*a*) *Conditions for Cultivation of Cells with Optimum Arsenite-oxidizing Activity*

(i) *Influence of Age of Culture.*—The arsenite-oxidizing activity per unit mass of cells was found to remain virtually constant for the first 3 days, and then to fall as the incubation period at 25°C increased. In most of the experiments cells were therefore harvested from young cultures, usually not more than 4 days old.

(ii) *Influence of Culture Medium.*—The most satisfactory medium we have used is the simple KA3 medium in which the carbon source is citrate and the nitrogen source is NH_4^+; the yield of cells is not as high as in other media examined, but their arsenite-oxidizing activity is higher. Although meat infusion broth containing 0·02M arsenite produces very heavy yields of cells, the enzymic activity per mg dry weight was only about 10 per cent. of those harvested from KA3 medium. In 1·5 per cent. Difco yeast extract solution containing 0·2 per cent. mixed KH_2PO_4-Na_2HPO_4 buffer salts for pH 7·4, 0·02 per cent. $MgSO_4.7H_2O$, and 0·02M $NaAsO_2$, a high yield of cells was obtained, but with only 40-50 per cent. of the relative activity; this medium, referred to as YA3, corresponds to KA3 in which NH_4^+ is replaced by more complex N sources. The same medium containing in addition 0·15 per cent. $NaNH_4HPO_4$ as in KA3 medium, also gave an excellent yield of cells, but the relative activity was only

30-35 per cent. It appears that the enzyme is formed in greatest amount when the N source is simple, as in NH_4^+, and that it is depressed by "richer" nitrogenous media.

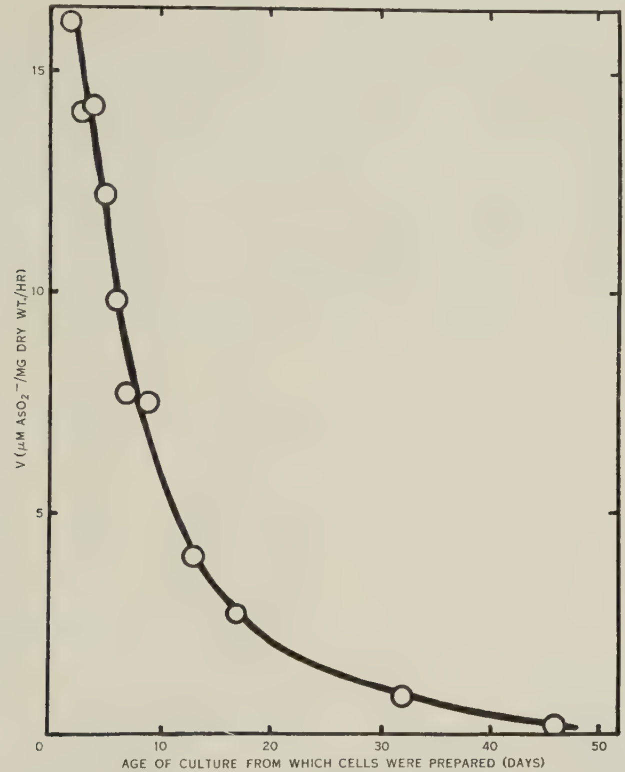

Fig. 1.—Variation of arsenite-oxidizing activity with age of the culture from which the cells were harvested.

(iii) *Adaptive Nature of the Enzyme.*—Cells grown in Koser medium without arsenite, or with 0·02M arsenate instead of arsenite, had no detectable initial arsenite-oxidizing activity, whether they were descended from cells routinely subcultured in the presence of arsenite or in its absence.

In some experiments it was observed that cells grown in the absence of arsenite developed slight activity after a lag period of 60 min or more. Although the post-lag development of activity was slight, it was about twice as great if the cultures yielding the cells had been sown with adapted cells.

These observations suggest the adaptive formation of enzyme, without cell multiplication, from reserve cell materials in the presence of substrate. When a small amount (10 per cent.) of Koser medium was added to the cell-buffer-arsenite system, the optical density doubled after 7 hr shaking at 20°C, indicating a twofold multiplication of cells, but the arsenite-oxidizing activity increased about 28 times compared with that of cells shaken in a system without added nutrient. This suggests adaptive enzyme formation during cell growth.

Cells grown in KA3 medium were equally active, whether the medium had been sown with strains continuously subcultured in medium containing arsenite, or not containing arsenite. This indicates the rapid adaption to arsenite oxidation.

(*b*) *Conditions for Optimum Activity of Washed Cells*

(i) *Suspending Buffer.*—The velocity of oxidation by cells washed repeatedly with distilled water was the same, whether they were suspended in 0·05M collidine buffer (Gomori 1946) or in 0·067M phosphate buffer at the same pH (6·4). Evidently an external source of phosphate is not required for arsenite oxidation. In the universal buffer of Teorell and Stenhagen as modified by Östling and Virtama (1946), which contains borate and citrate in addition to phosphate, the initial velocity was likewise the same; after prolonged contact, however, the velocity increased, probably through utilization of citrate followed by consequent cell multiplication, with increased enzyme formation in the presence of arsenite.

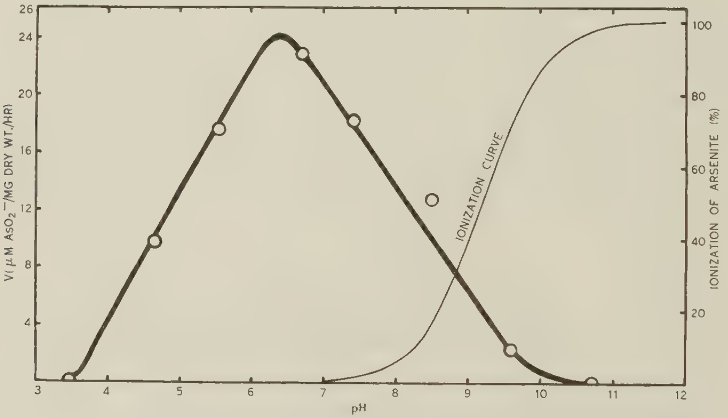

Fig. 2.—Variation of arsenite-oxidizing activity with pH, compared with the dissociation curve of $HAsO_2$.

(ii) *pH Optimum.*—This was determined from the initial rates of oxidation of 0·02M arsenite by aerated washed suspensions in Teorell and Stenhagen's universal buffer, at virtually constant ionic strength, over the pH range 3-11 at 20°C. The reaction was followed by iodometric titration. The values obtained when velocity was plotted against the pH are illustrated in Figure 2, from which it is evident that under these conditions there is a fairly sharp optimum at pH 6·2-6·6. The suspensions held at extreme pH's in these experiments were not tested for viability; but the organism is able to grow in the presence of arsenite between pH 9·5 and 4·5, the growth being very slow at the acid pH. The pH optimum was not significantly altered when the cells tested were harvested from arsenite-containing media in which heavy buffering maintained different pH's during growth.

On the same graph is plotted also the percentage of ionized molecules of sodium arsenite, calculated from the Henderson equation with the pK_a of arsenite as 9·22. It is seen that at pH values up to optimum, virtually all the arsenite exists as undissociated molecules. However, since the activity falls off beyond the optimum pH more rapidly than can be accounted for by the ionization of arsenious acid, the drop of activity is therefore primarily due to effects on the enzyme system.

The same pH optimum was found when the oxygen uptake during arsenite oxidation by washed cell suspensions was measured manometrically.

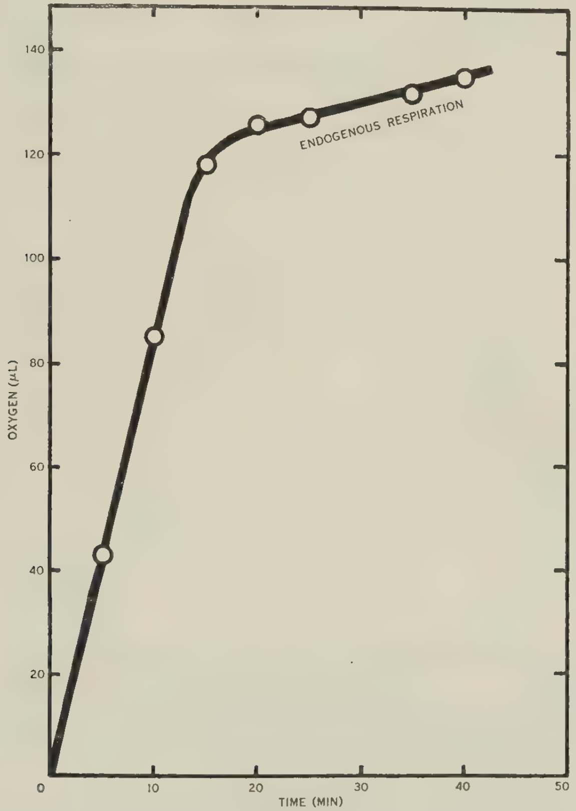

Fig. 3.—Time course of oxidation of 5×10^{-4}M arsenite.

(iii) *Substrate Concentration.*—Figure 3 indicates the course of oxygen uptake when $4 \cdot 3 \times 10^{-3}$M arsenite was oxidized by cells. The velocity of oxidation fell off appreciably only after 90 per cent. of the arsenite had been oxidized.

Figure 4 indicates the rate of oxygen uptake measured immediately after tipping various quantities of arsenite into the vessels. Up to a concentration of 9×10^{-4}M arsenite the rate was virtually a linear function of concentration and at that molarity it appeared to reach a limiting velocity. Half-maximum rate was reached when the concentration of arsenite equalled $4 \cdot 5 \times 10^{-4}$M. Even at the lowest substrate concentrations, however, rates of oxidation were linear almost to completion, a fact which is unexpected if the relation between rate and substrate concentration were to be interpreted according to the Michaelis-Menten equation. Examination of the data by the methods of Lineweaver and Burk also indicated that they could not be interpreted in this way. This situation, and the above-mentioned linearity of oxygen uptake almost to completion of arsenite oxidation at all the concentrations tested, suggest that the results are complicated by diffusion processes through the cell wall.

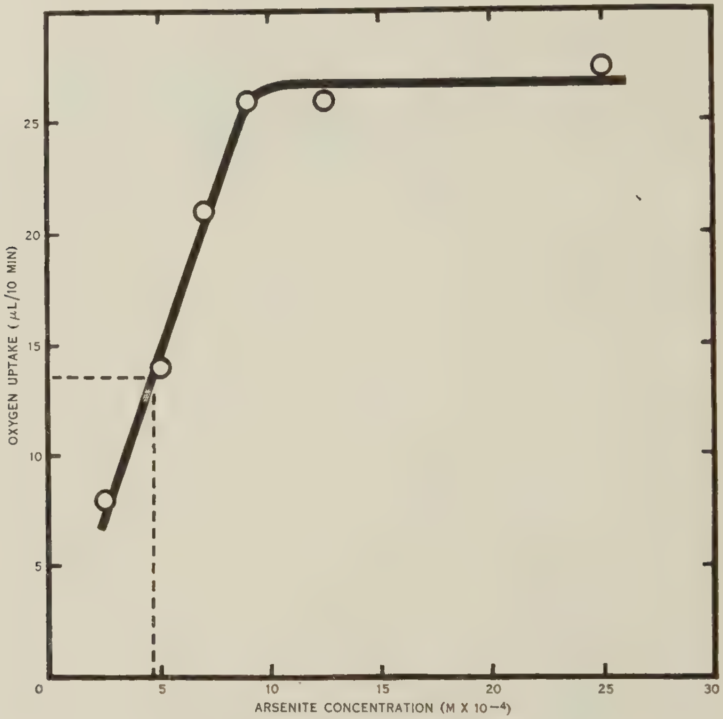

Fig. 4.—Variation in rate of oxygen uptake with the concentration of substrate.

Measurement of the relationship between substrate concentration and rate of arsenite oxidation by iodometric methods gave the same half-maximum rate.

(iv) *Temperature*.—The data in Figure 5 show the dependence of arsenite oxidation rate on temperature. Three suspensions were used, the results being adjusted to the same level of activity (Wilson 1938). The oxidation was followed by titration. For temperatures less than the optimum, the velocity remained constant for 60-80 min, but beyond the optimum it decreased noticeably after 30 min. From Figure 5 it is seen that under these conditions the optimum temperature was near 40°C. The Q_{10} between 20 and 30°C was found from the calculated best-fit line to be 2·4, and between 30 and 40°C, 1·6.*

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* In the preliminary note, these Q_{10} values, measured from a visual-fit line, were given as 1·73 and 1·43, respectively.

Of the various equations commonly used to describe the dependence of velocity of biological reactions on temperature, those of Belehrádek (1935) and of Arrhenius were tested. The data are graphed appropriately in Figures 6 and 7 respectively. Statistical analysis shows that the latter method (temperature characteristic $b = 1{\cdot}27 \pm 0{\cdot}08$) gives a better fit of the data than the former (apparent energy of activation = 7750 ± cal/mole).

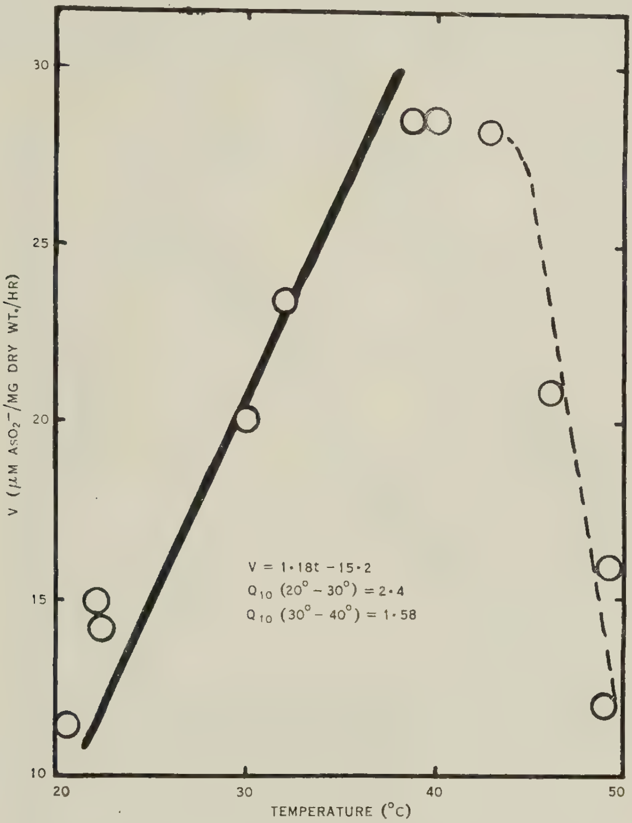

Fig. 5.—Variation of velocity of arsenite oxidation with temperature.

(*c*) *Other Acceptors*

In addition to reducing oxygen in the presence of arsenite, washed adapted cells are able to reduce suitable dyes under anaerobic conditions.

It is necessary to use, as electron acceptors, redox indicators with E_0 values appropriately higher than that of the arsenite-arsenate system itself (E_0' at pH $7{\cdot}0 = +0{\cdot}077$ to $+0{\cdot}167$ V, depending upon which of the several values for E_0 recorded in the literature is accepted). Suitable acceptors are indophenol-2,6-dichlorophenol, phenol blue, and *m*-carboxyphenol-indo-2,6-dibromophenol (E_0' at pH $7{\cdot}0 = +0{\cdot}217$, $+0{\cdot}224$, and $+0{\cdot}250$ V, respectively). With indophenol-2,6-dichlorophenol, arsenite dehydrogenase has an optimum around pH 4·5.

Ferricyanide is also able to oxidize arsenite anaerobically in the presence of washed cells. The reaction was followed in Warburg manometers by measuring CO_2 evolution from bicarbonate buffer, as a result both of the arsenate and of ferrocyanide formed in the reaction. Anaerobiosis was obtained with a small stick of yellow phosphorus in the centre well. Under these conditions, arsenite was oxidized at only one-third the rate at which it is oxidized by air.

These results, which show the existence of an arsenite dehydrogenase able to react with a number of acceptors, are considered further in Paper III of this series.

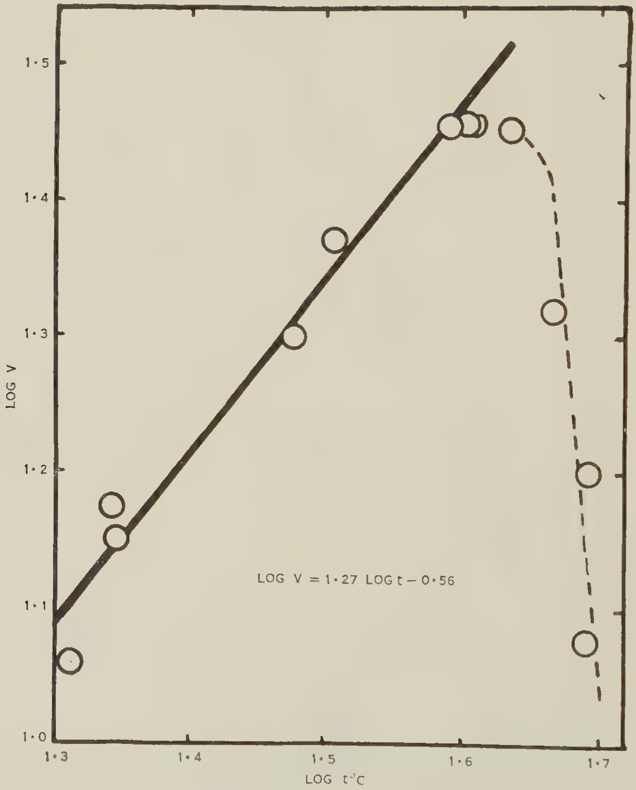

Fig. 6.—Variation of log velocity of arsenite oxidation with log temperature (°C).

(*d*) *Effect of Inhibitors on Arsenite Oxidation by Intact Cells*

(i) *Cyanide and Azide.*—In view of the high redox potential of the arsenite-arsenate system, these inhibitors were used to test for the presence of iron or copper oxidases.

When the rate of arsenite oxidation was measured by iodometric titration, any cyanide or azide present was first removed by acidifying and boiling the suspension.

When cyanide was added at the same time as the arsenite, it was observed that the extent of inhibition was greater in the second half hour than the first. Complete inhibition was produced by 10^{-3}M cyanide after this time. This was not observed when the cell suspension was left in contact with cyanide for 30 min, and then diluted with an equal volume of cyanide-free arsenite.

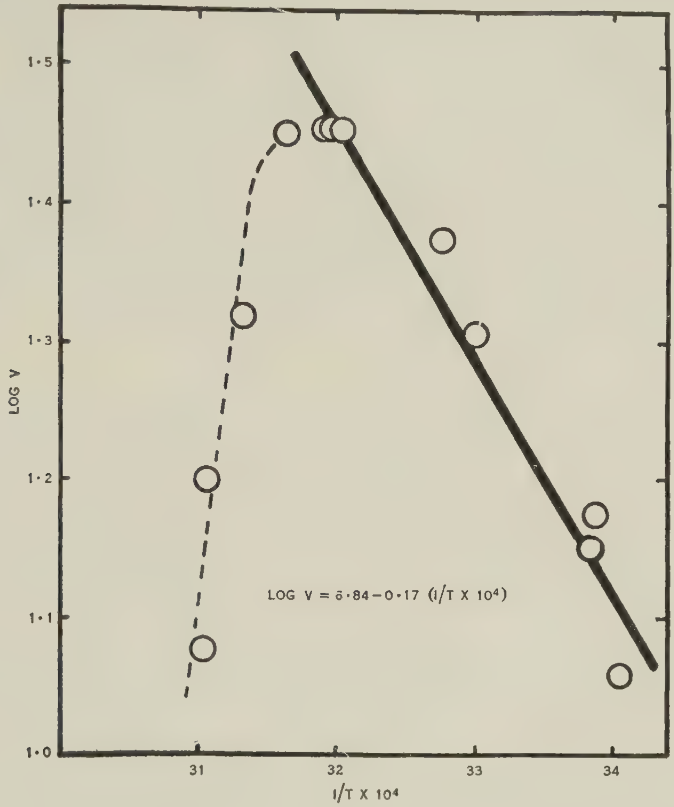

Fig. 7.—Variation of log velocity of arsenite oxidation with $1/T$.

Table 1

INHIBITION OF ARSENITE OXIDATION BY CYANIDE AT 23°C

Strain 17, original

Cyanide (final molarity)	Arsenite Oxidized (μM/mg dry wt./hr)	Inhibition (%)
0	31·0	—
10^{-4}	7·50	75·8
10^{-5}	17·8	42·6
10^{-6}	27·8	10·3

Fifty per cent. inhibition was thus produced by between 10^{-4} and 10^{-5}M cyanide under these conditions.

Sodium azide, tested at a concentration of 0·01M, completely inhibited oxidation.

(ii) *Carbon Monoxide Inhibition.*—Carbon monoxide is known to combine with, and to inhibit, cytochrome oxidases as well as the copper oxidases. In the first case, the CO compounds are typically dissociated by light, whereas in the second they are not. Although a typical light-reversible inhibition was observed 6 months after the strain was isolated, suspensions prepared 22 months later reacted differently. The experiments are therefore reported in some detail.

In the earlier experiments, suspensions of strain 17 were equilibrated with relevant gas mixtures, and were placed in rubber-stoppered 50-ml standard flasks, of plain or ruby glass, which had been flushed out with the gas mixture. When aliquots were removed for iodometric titration, the flushing was repeated. The light reversal of the inhibition was obtained either by diffuse daylight or by exposure of the flasks to the concentrated illumination of a battery of flood lights, the beam reaching the flasks in a glass constant-temperature bath after passing through a cooling cell.

TABLE 2

INHIBITION OF ARSENITE OXIDATION BY CO, AND ITS REVERSAL BY LIGHT

Strain 17, original

Experiment	Cell Concentration (mg dry wt./ml)	Temperature (°C)	Light	CO/O_2 or N_2/O_2	Velocity of Oxidation (μM AsO_2^-/mg dry wt./hr)	Inhibition (%)	Light Reversal (%)
1	0·13	23	Diffuse daylight	4	L 20·2	16·8	27
					D 18·7	23·0	
					C 24·3	—	
2	0·13	22	960 f.c.	19	L 18·0	26·6	34
					D 14·7	39·7	
					C 24·4	—	
3	0·35	1	960 f.c.	19	L 3·47	31·6	39
					D 2·41	51·8	
					C 5·00		

L = exposed to light; D = protected from light by ruby flask; C = control. L and D in presence of $CO+O_2$; C in presence of N_2+O_2.

In view of the finding of Kubowitz and Haas (1932) that the light sensitivity of carbon monoxide inhibition of respiration of vinegar bacteria and of yeasts was 4½ times greater at 0°C than at 10°C, some experiments were carried out in water-baths containing an excess of shaved ice. Because of the greatly reduced velocity of oxidation at this temperature, much thicker cell suspensions were then required. Rates of arsenite oxidation were measured over time intervals such that the amount of dissolved oxygen was not significantly lessened during the experiment.

Typical experiments are summarized in Table 2 which demonstrate (*a*) that carbon monoxide inhibited arsenite oxidation, the inhibition being greater in a mixture of 95 per cent. CO and 5 per cent. O_2 than in a mixture of 80 per cent. CO and 20 per cent. O_2; (*b*), that although inhibition was greater at 1°C than at 22°, light reversal of inhibition at 1°C was only slightly, if at all, greater than at 22°C.

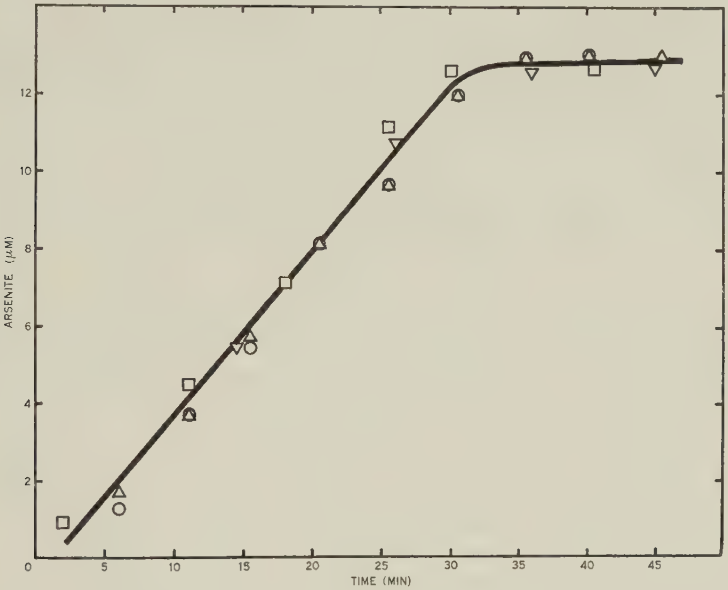

Fig. 8.—Time course of arsenite oxidation in presence and absence of carbon monoxide, measured by oxygen uptake and by iodometry. Substrain *L*2.

The effect of carbon monoxide was re-examined after substrain 17*L*2 had been isolated in the course of later experiments (Legge and Turner 1954). When arsenite oxidation was measured manometrically at 38°C, reproducible inhibition of oxygen uptake by carbon monoxide in the dark with CO/O_2 ratios up to 30 to 1, could not be obtained; in some cases, indeed, there was a slight acceleration. Although it had been previously observed by Fujita and Kodama (1934) that carbon monoxide did not inhibit the respiration of pseudomonads, the failure to confirm our earlier findings was unexpected. Further experiments were therefore performed in order to throw some light on this capricious phenomenon.

Impurities in the carbon monoxide, which was prepared either by dehydrating formic acid with concentrated sulphuric acid or by heating calcium carbonate and zinc, were considered to be most unlikely; indeed, samples of bakers' yeast showed a typical inhibition, with some light reversal, and with complete reversibility when the CO/N_2 mixture was replaced by O_2/N_2 mixtures.

The possibility that carbon monoxide might be oxidized by the organisms, and thus conceal an inhibited oxygen uptake due to arsenite oxidation, was considered. No evidence was found to support the hypothesis of carbon monoxide oxidation, and an experiment in which arsenite disappearance was followed iodometrically in parallel with oxygen uptake showed that arsenite oxidation by the *L*2 substrain was insensitive to CO inhibition by either method. At given periods the vessels were removed from the manometers, solid sodium carbonate was added, and the remaining arsenite was titrated iodometrically; the titration was completed within 2 min of removing the vessels. The results are set out in Figure 8.

It can be seen that, whether measured by oxygen uptake or by iodometric titration, the disappearance of arsenite was not influenced by the presence of CO, the CO/O_2 ratio being 16·5.

TABLE 3

COMPARISON OF EFFECT OF CO ON ARSENITE OXIDATION BY *L*2 CELLS IN PHOSPHATE BUFFER, AND IN BICARBONATE BUFFER

Gas Mixture	Arsenite Oxidation (μM AsO_2^-/mg N/hr)	
	Phosphate Buffer*	Bicarbonate Buffer†
3·5% O_2/CO	37·8	36·5
3·7% O_2/N_2	42·2	35·8
21% O_2/N_2	58, 60·8	42·1

* $3\cdot5\times10^{-3}M$, pH 7·4.

† $3\cdot5\times10^{-3}M$ HCO_3^- with $3\cdot5\times10^{-4}M$ CO_2, pH 7·4.

Since Warburg (Warburg, Kubowitz, and Christian 1931) had recorded instances in which the CO inhibition of respiration in animal tissues required the presence of CO_2/bicarbonate, and failed in the presence of NaCl-phosphate buffers, the experiments were repeated in the presence of CO_2. These were rendered difficult because of two opposing requirements: high buffering capacity was required in order to protect the system against the strongly acid oxidation product, arsenic acid, and, at the same time, high partial pressure of CO was required, thus limiting the partial pressure of CO_2. The method of Pardee (1949) was used; the diethylamine-carbonate buffer system was chosen to give a partial pressure of $1\cdot5\times10^{-2}$ atm CO_2. With $3\cdot5\times10^{-4}M$ dissolved CO_2 and $3\cdot5\times10^{-3}M$ HCO_3^-, it was calculated that the initial pH was 7·3. Since its buffer capacity was low, only the initial rate of arsenite oxidation was considered.

The results with a suspension of KA3 cells previously stored for 60 days in the refrigerator, but still highly active, are shown in Table 3. For comparison, arsenite oxidation was measured, in the presence of KOH papers, in phosphate buffer pH 7·4, of the same molarity as the HCO_3^-. In order to estimate the oxygen affinity of the cell oxidase, the measurements of uptakes in these two buffers were also carried out in air. The temperature was 38°C.

Slight CO inhibition (10 per cent.) was observable in the phosphate buffer, but the results in bicarbonate buffer showed no significant difference. Since the CO/O_2 ratio of 25 in the above experiment exceeded the ratio of 20, which in earlier experiments was found to produce 50 per cent. inhibition of arsenite oxidation, and since 10 per cent. inhibition was, with one exception, the highest found in the present series, it is apparent that a substantial change in the characteristics of the oxidase had taken place. Since a significant decline in oxidation rate at the low partial pressure of oxygen was observed in both buffers, it is apparent that the failure to find CO inhibition was not due to an abnormally high affinity of the oxidase for oxygen.

The insensitivity of the *L*2 substrain to carbon monoxide inhibition was observed with substrates other than arsenite; with formate, for example, which is oxidized readily, one experiment with CO/O_2 ratio of 4 showed 10 per cent. inhibition. An experiment in which the oxidation of *p*-phenylene diamine was measured at 0°C showed no inhibition with 50 per cent. CO in O_2.

TABLE 4

FAILURE OF CO INHIBITION OF ARSENITE OXIDATION BY (*a*) *L*2 CELLS, CONTRASTED WITH INHIBITION OF ASCORBATE OXIDATION BY (*b*) *L*2 CYTOCHROME OXIDASE AT 38°C

Treatment	Oxygen Uptake (μl O_2/hr)		Inhibition (%)	
	(*a*)	(*b*)	(*a*)	(*b*)
N_2/O_2	194	276	—	—
CO/O_2 light	192	194	0	30
CO/O_2 dark	192	134	0	51

In the whole series, only one experiment showed a typical CO inhibition. This was carried out with KA3 cells of the *L*2 substrain, 5 wk old, in phosphate buffer pH 6·8, 38°C, with CO/O_2 or N_2/O_2 ratios of 10. One vessel containing CO was irradiated with a 300 W "Hanovia" u.v. lamp through a window in the bath. In N_2/O_2, arsenite oxidation proceeded at 131 μM/mg N/hr; the rate was inhibited 29 per cent. in CO/O_2 in the dark, and only 15 per cent. when irradiated. When a sample of cells of the 17*X* substrain, cultivated in KA3 medium, was tested under conditions identical with the above, an inhibition of 19 per cent. was observed in the dark, with negligible light reversal.

Although the arsenite-oxidizing activity of the 17*L*2 substrain was almost completely insensitive to carbon monoxide inhibition, a cytochrome oxidase preparation from this substrain (cf. Legge and Turner 1954), as well as from the 17*X* substrain, was more readily inhibited. Table 4 sets out the results with oxidase AA, ascorbic acid, and 2,6-dichlorophenol indophenol, at 37°C, CO/O_2 and N_2/O_2 ratios 11·5. For comparison, 17*L*2 cells oxidizing arsenite were included. Vessels were irradiated from below by light reflected from a 250 W u.v. low-pressure lamp, control vessels being covered with metal foil.

It can be seen that the activity of the cytochrome oxidase was decreased 50 per cent. by CO in the dark, and only 30 per cent. when illuminated. The oxidase recovered its original activity on replacement of the CO/O_2 mixture by N_2/O_2.

(iii) *Other Enzyme Inhibitors.*—Experiments with other enzyme inhibitors were less informative than those with cyanide and carbon monoxide. Thus fluoride, which inhibits Ca^{++} or Mg^{++} activated enzymes, as well as the cytochrome system (cf. Borei 1945; Slater and Bonner 1952), inhibited arsenite oxidation only 21 per cent. after being in contact with cells for 90 min (0·01M NaF, pH 6·4-7·0, 20°C). With 0·1M NaF the inhibition increased with time, reaching 67 per cent. after 100 min. Under the same conditions, 0·02M pyrophosphate produced only 16 per cent. inhibition.

Other inhibitors of mental enzymes, such as 0·001M α,α'-dipyridyl (for Fe^{++}) or diethyldithiocarbamate (for Cu^{++}) were without effect. The sulphide ion, which could not be used in presence of arsenite because of arsenious sulphide formation, had a negligible effect when cells exposed to 0·001M concentration for 15 min were subsequently tested.

Neither urethane (0·1M) nor the well-known $-SH$ enzyme inhibitor iodoacetate (0·01M) inhibited the arsenite oxidation.

In view of the likelihood that the terminal oxidation of arsenite takes place on the cytochrome system, the effect of malonic, succinic, and fumaric acids was tested. Although only the cytochromes of higher redox potential were likely to be involved in the arsenite oxidation, the possibility was recognized that electron transport from arsenite might have to compete with that from other substrates.

At pH 6·4, when malonic acid is present mainly as the malonate ion, no significant inhibition was observed. At pH 4, however, 30-40 per cent. inhibition was observed with 0·05M malonate which was overcome after 40-60 min; 0·05M succinate and fumarate behaved similarly, and the effect of malonate added together with fumarate was simply to prolong the period of inhibition; after 80 min, arsenite oxidation proceeded equally rapidly in the presence of any of these acids.

These experiments were carried out in Universal buffer pH 4·5, which contains citrate. On repeating in 0·05M phosphate buffer, the initial rates in the presence of the above dicarboxylic acids were greater than the control rate. Between 20 and 60 min, the arsenite oxidation rate slackened, and then approximated to the original. Since the opacity of the suspension increased during the experimental period in comparison with the control, it appears probable that interference with arsenite oxidation is complicated by secondary adaptive phenomena related to the contact between the KA3-trained cells and a new, assimilable carbon source (cf. Turner 1954). The initial effects observed may indeed be of a non-specific character owing to the pH regulating mechanism of the cell being overwhelmed by a permeable acid.

(*e*) *Stability of Enzyme System*

The cell suspensions were inactivated by drying or by acetone treatment. Addition of 0·5 per cent. toluene was followed by a distinct clearing of the suspension, and lysis of many cells. Such treated suspensions no longer oxidized arsenite.

IV. DISCUSSION

Experiments on cell-free arsenite dehydrogenase, and on the cytochrome oxidase of the organism, are reported in subsequent papers. This communication is limited to experiments with intact cells; hence restrictions due to permeability must be recognized, particularly when the activity of possible inhibitors is considered.

The significance of the value obtained for the apparent energy of activation ($\mu = 7750$) calls for discussion. Dehydrogenase reactions probably do not limit the reaction at conditions approaching the optimum, since the characteristic values for dehydrogenases are of a higher order; thus for succinic dehydrogenase of bacteria, $\mu = 16{,}700$; for dehydrogenase of *B. coli,* $\mu = 19{,}400$; and for heart dehydrogenase-cytochrome-cytochrome oxidase, $\mu = 16{,}000$ (Sizer 1943). It is equally unlikely that the cytochrome system is responsible for the low value, since, although Hadidian and Hoagland (1940) obtained $\mu = 9500$ for oxidation of phenylene diamine by cytochrome oxidase, the cytochrome-cytochrome oxidase reaction has been found to have a μ value of 16,000; indeed, in the succinic oxidase system, for which $\mu = 11{,}000$, succinic dehydrogenase is the limiting reaction (Stearn 1949). Our finding of 7750 cal/mole for arsenite oxidation is, therefore, less than values which others have found for oxidations; on the other hand, it lies in the range found for diffusion processes (Barrer 1949). This is consistent with the evidence based upon the relationship between arsenite concentration and rate of oxidation.

In view of the rapidity with which many microorganisms are able to modify their protoplasm in response to changes in the environment, the use of washed cells, under conditions which may be made to approach "physiological conditions" closely, may render difficult the interpretation of inhibitor data in simple enzymic terms. This may be so with cyanide, with which the degree of inhibition changed with time. The observations of Chin (1950) and of Ephrussi and Slonimski (1950) on the rapidity with which yeast is able to alter its cytochromes in response to changes in partial pressure of oxygen in a nitrogen-free medium suggest that such an interpretation is worthy of further experimental investigation. The same may be so with the inhibition observed with dicarboxylic acids. Here, however, the process may be complicated by competition between two oxidizable substrates for the same oxidase, and by the possibility that the cells may be adapting to these carbon sources during the course of the experiment.

The variable results obtained when measurements of the carbon monoxide inhibition were made should be noted. Since samples of bacterial oxidase with differing spectroscopic properties (cf. Legge 1954) show carbon-monoxide inhibition of leuco-indophenol oxidation, the failure of the strains from which these oxidases were derived to show significant carbon monoxide inhibition of arsenite oxidation cannot be due to the lack of a cytochrome component. Since fresh samples of packed cells, as well as samples which had been stored in the refrigerator for periods up to 60 days, showed similar behaviour, pathological changes in the stored cells cannot be implicated. The possible significance of the buffer used, whether bicarbonate or phosphate, has been excluded by experiment, and the agreement between arsenite disappearance measured by oxygen uptake or iodometric titration excludes significant carbon monoxide oxidation. It would appear most unlikely that the insensitive cells differed from the sensitive cells in the permeability of their walls to such a small uncharged molecule as that of carbon monoxide.

We can only conclude that the change in carbon monoxide sensitivity of the arsenite oxidation must be due to altered reactivity of the cytochromes within the cell. The fact that most of the substrains used in the manometric studies had less arsenite-oxidizing activity than the earliest substrains isolated, in which undoubted carbon monoxide inhibition was observed, is confirmatory evidence that additional changes in the organism, which we have been unable to probe experimentally, have taken place during the period of the investigation.

The adaptive character of the arsenite dehydrogenase makes it a particularly interesting enzyme to investigate. So far, our studies of the conditions for the cultivation of maximally active cell suspensions have shown that the enzyme system is less active in richer culture media than in those containing inorganic nitrogen and sulphur, with citrate as the only carbon source. The variation in activity reported in the succeeding paper shows that other factors must be taken into consideration, chief among these being the cultivation of cells with a high concentration of cytochrome components with high redox potential. This is now being reinvestigated.

V. ACKNOWLEDGMENTS

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BACTERIAL OXIDATION OF ARSENITE

III. CELL-FREE ARSENITE DEHYDROGENASE

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Summary

A soluble, cell-free arsenite dehydrogenase is liberated from cells of strain 17 of the arsenite-oxidizing pseudomonad (*Pseudomonas arsenoxydans-quinque*) after grinding with powdered glass. The enzyme is moderately stable, retaining its activity for considerable periods on storage at low temperatures. It may be dialysed without loss of activity, and withstands salting out.

Its activity may be measured in the presence of suitable electron acceptors.

In the course of a prolonged series of enzyme preparations, the yield dropped considerably. This was traced to variation in the strain, and substrains were isolated from which original yields of enzyme were obtained.

No evidence was found that the activity of the enzyme was dependent on accessible $-SH$ groups.

I. Introduction

Previous papers (Turner 1949, 1954; Turner and Legge 1954) gave a detailed description of arsenite-oxidizing bacteria, defined the conditions for obtaining active adapted cells, described their arsenite-oxidizing characteristics, and referred briefly to arsenite dehydrogenase activity in extracts of disrupted cells. The present paper continues the study of the dehydrogenase obtained by disrupting cells with powdered glass. A later paper (Legge 1954) describes the reconstruction of oxidizing systems from disrupted cells.

II. Methods

(*a*) *Routine Preparation of Enzyme*

Cells for preparing arsenite dehydrogenase were usually harvested from 3-day cultures at 25°C in YA3 medium (Turner 1954), the mean yield being 6·3 g packed cells per l. For special purposes, 6-day cultures in KA3 medium (Turner 1954) were used, the mean yield being 0·93 g packed cells per l.

Cells were harvested in a Sharples supercentrifuge; they were resuspended, and were finally centrifuged to provide 12-25 g of bacterial paste. After storing at 4°C, usually for 5-8 days, the cells were ground in a chilled Utter-Werkman mill with twice their weight of powdered Pyrex glass and twice their weight of M/15 pH 7·2 phosphate buffer. The effluent was then spun at 3500 r.p.m.

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in a Wifug angle centrifuge for 30 min, thus separating the glass and cell debris (P), and the few undamaged cells, from the supernatant solution (S_1). Fraction S_1 contained half to two-thirds of the total arsenite dehydrogenase which could be extracted by further washing of fraction P, and was generally kept separate from the subsequent washings (S_2, S_3). After two washings, fraction P contained only negligible arsenite dehydrogenase activity, although it contained the greater part of the bacterial cytochromes.

S_1, generally a dirty yellowish turbid solution, was then subjected to a further fractionation in the high-speed angle head of the I.E.C. Model 2 refrigerated centrifuge, which was operated to maintain the same temperature as the room. The average gravitational field to which the solutions were subjected was calculated to be 19,000g. After 60 min spinning, a small translucent pink pellet was thrown down (S_1P), leaving a relatively clear supernatant, which was then stored at -5°C with a few drops of toluene as preservative.

(*b*) *Measurement of Enzyme Activity*

Dehydrogenase activity was estimated from the rate of reduction of 2,6-dichlorophenol indophenol in Thunberg tubes, or manometrically, from the rate of evolution of CO_2 when ferricyanide was used as the electron acceptor in bicarbonate buffer. Nitrogen was determined by micro-Kjeldahl, a Markham still being used.

The unit of dehydrogenase activity $= 1/t \times 10^5$, when $t =$ the time in minutes for 90 per cent. reduction, at 25°C, of 0·3448mM 2,6-dichlorophenol indophenol by 0·1 ml enzyme in 0·004M arsenite, the volume being made up to 5 ml with buffer. For example, a sample which took 18·2 min to bring about reduction of the dye under those conditions was said to contain 5500 units per ml; this is equivalent to an oxidation of 26·0μM arsenite per ml per hr. The same sample of enzyme, when assayed manometrically with 2×10^{-2}M ferricyanide as acceptor, oxidized arsenite at approximately half this rate.

III. Results

(*a*) *Partition of Arsenite-Dehydrogenase Activity between Cell Juice and Cell Particles*

The extent to which high-molecular-weight particles were removed from the S_1 fractions by this treatment varied in different batches of cells, and could not be correlated with the other changes discussed below. In some cases the supernatant from the first centrifugation of the disrupted cells showed only a faint Tyndall effect, and little or no further clarification after high-speed spinning. In other cases, the supernatant after 60 min at high speed showed the presence of a high-molecular-weight component which was concentrated in the lower part of the centrifuge tubes. In nine out of 12 preparations in which the enzyme activity of both upper and lower fractions was measured, the lower showed 7-17 per cent. greater activity. In one instance, the lower fraction was significantly (12 per cent.) less active than the upper.

Since all the enzyme remains in solution after low-speed centrifugation, it is concluded that 17 per cent. represents the maximum amount of enzyme remaining bound to relatively high-molecular-weight particles, and that most exists as a true protein solution. No correlation could be observed between the amount of arsenite dehydrogenase bound to high-molecular-weight particles, and the enzyme activity of the S_1 fractions. The relationship, if any, between the slowly sedimenting, high-molecular-weight particles carrying bound arsenite dehydrogenase, and the pink or buff cytochrome-rich pellets sedimenting more rapidly in the high-speed head, was not investigated in detail. The finding that the cytochrome-rich pellet may be washed free of arsenite dehydrogenase suggests that the particles may be different.

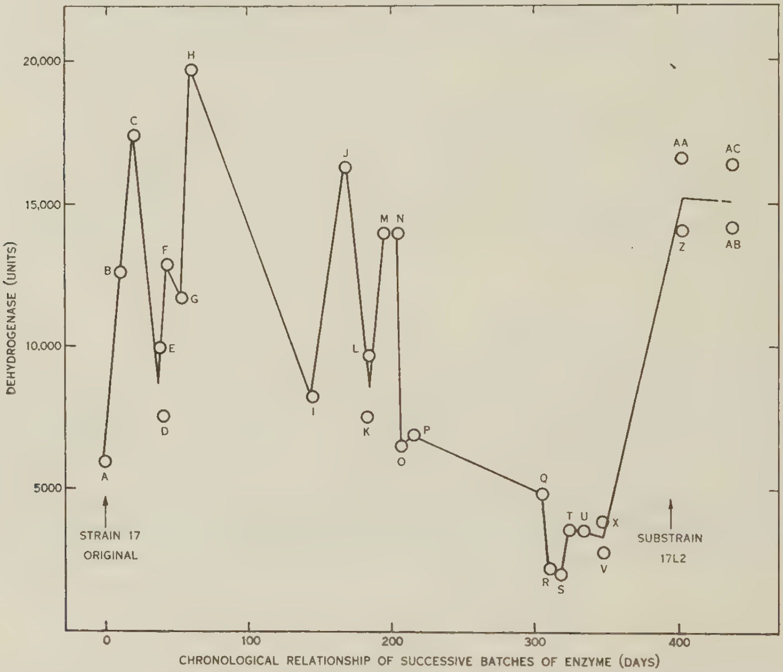

Fig. 1.—Secular variation in activity of 27 successive batches of arsenite dehydrogenase.

(*b*) *Secular Changes in Enzyme Yield*

When the procedure for fractionating the enzymes had been put on a routine basis, fresh preparations were made at intervals in order that stocks of enzyme could be accumulated.

Storage of packed washed cells at 4°C outside the limits of 5-8 days was rare, and appeared unrelated to the gross changes in enzyme yield found to occur. These are set out in Figure 1, and represent the arsenite dehydrogenase activity in the first extract (S_1) from the crushed cells. Essentially the same picture is obtained when the enzyme content of the second and subsequent extractions of the crushed cells is taken into account. The data must only be considered as semi-quantitative, since it was not always possible to assay the enzyme activities of the extracts immediately after preparation, but deterioration at 4°C is usually slow (cf. Fig. 2).

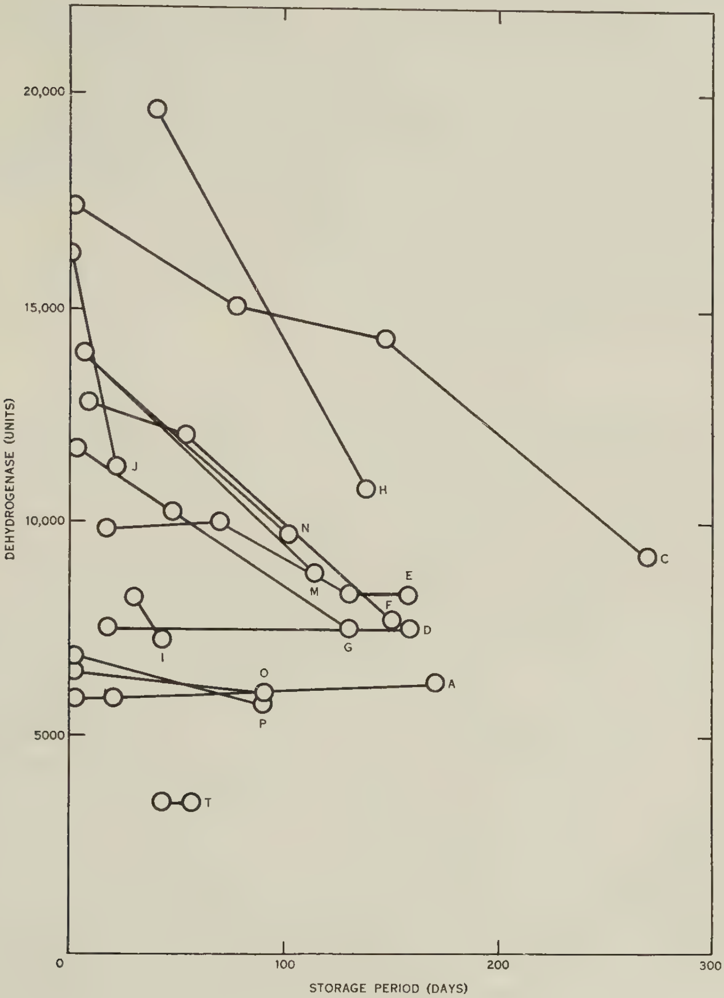

Fig. 2.—Stability of arsenite dehydrogenase on storage between −5°C and +1°C.

Until mid 1949, the activity of the arsenite dehydrogenase varied between 6000 and 17,000 units in S_1; the succeeding seven preparations, however, gave extracts whose activity varied from 2000 to 5000 units. This was correlated with a drop in arsenite-oxidizing activity of the whole cells: cultures from which high yields of enzyme had been obtained were able to oxidize arsenite at rates varying from 70 to 140μM/mg N/hr, measured manometrically, whereas cells from which enzymes *Q* to *X* had been prepared oxidized only 13-20μM/mg N/hr. The low yields of enzyme were, therefore, not due to incomplete extraction, but to a lowered production by the cell. The activity remained low not only in YA3, but in KA3, even when this was enriched with iron.

The strain then in use was then plated out, and separate colonies were subcultured in arsenite-Koser medium to which brom-phenol blue had been added (Turner 1954); substrains were then selected according to the speed of production of acid. In this way two substrains were isolated, 17*X* and 17*L*2, the former cells being able to oxidize arsenite at the rate of approx. 30μM/mg N/hr and the latter, approx. 100μM/mg N/hr. The secular change in enzyme yield from crushed cells is explained, therefore, by the transformation of the original strain 17 into the two variants 17*X* and 17*L*2, the former of these showing more rapid growth in the media used.

Like the original strain (Turner 1949), the arsenite dehydrogenase activity of both the new substrains is adaptive.

(*c*) *Characteristics of Crude Enzyme Solution*

So far, purification of the enzyme present in the crude extracts has not been attempted. The following observations refer, therefore, to enzyme solutions of unknown purity, and, probably, in view of the alterations observed in the strains of organisms used, of unknown homogeneity.

The stability of the enzyme appears maximum at about pH 6. When solutions were acidified, no change occurred down to pH 4·8, but at 4·2 the enzyme was totally precipitated. After standing at 23°C for 24 hr, the enzyme could be redissolved by adjusting the pH, over 70 per cent. of the activity being recovered. Some samples were stable to dialysis, whereas others lost up to 30 per cent. of their activity.

Dilute enzyme solutions have been observed to increase in activity on standing 24 hr at room temperature; some material precipitated out under these conditions.

Solutions of arsenite dehydrogenase stored between -5°C and $+1$°C in presence of toluene were periodically sampled. The data set out in Figure 2 show that the enzyme may be classed as relatively stable, and that the initial level of activity has no significant effect on the keeping quality. The one sample of enzyme (*T*) prepared from variant *X* appears to show stability of the same order as solutions obtained from earlier cultures.

(*d*) *Search for Inhibitors of Arsenite Dehydrogenase*

A number of enzyme inhibitors have been examined for influence on arsenite oxidation by intact cells (Turner 1949; Turner and Legge 1954). The most effective were those whose point of attack was on the oxidase end of the system. The separation of the dehydrogenase from the cells enabled further inhibitors to be examined without regard to the limitations of permeability, inseparable from experiments on intact cells.

Thunberg techniques were used since, in the presence of the dehydrogenase, arsenite reduces 2,6-dichlorophenol indophenol more readily than ferricyanide. A number of enzyme preparations were used. Substances which inhibited arsenite dehydrogenase activity of the clarified cell juice are listed in Table 1, and those which did not inhibit, or brought about an acceleration, are listed in Table 2.

TABLE 1

SUBSTANCES WHICH INHIBITED ARSENITE DEHYDROGENASE ACTIVITY OF CLARIFIED CELL JUICE

Enzyme	Substance	Molarity	Incubation Period	Activity $\frac{t\text{ control}}{t\text{ inhibitor}} \times 100$
19.x.48	Arsenate*	$4 \cdot 4 \times 10^{-3}$	Nil	93
		$4 \cdot 4 \times 10^{-2}$	Nil	86
19.x.48	Cu^{++}*	2×10^{-5}	Nil	96
		2×10^{-4}	Nil	75
		2×10^{-3}	Nil	33
N†	Hg^{++}	10^{-3}	—	94
	Cu^{++}	10^{-3}	—	58
V†	Hg^{++}	10^{-3}	—	89
	Cu^{++}	10^{-3}	—	35
T‡	*p*-Chlormercuribenzoate	10^{-3}	1 Hr§	46
M‡	*p*-Chlormercuribenzoate	$10^{\ 3}$	5 Hr§	55

* pH 5·9, buffer.

† Arsenite 20μM, indophenol 1μM, volume 2·5 ml, veronal buffer, pH 6·8.

‡ Arsenite 20μM, indophenol 1μM, volume 2·5 ml, phosphate buffer, pH 6·8.

§ Dialysed 16 hr.

No inhibition was observed when *p*-chlormercuribenzoate or *o*-iodobenzoate was added with the substrate, or was incubated for 15-20 min with enzyme before addition of arsenite. With the exception of Cu^{++}, therefore, none of the reagents reacts immediately with groups in the enzyme. The inhibition found with *p*-chlormercuribenzoate is apparently the result of a slower reaction which forms a compound stable to dialysis.

In a number of instances, the addition of substances examined for possible inhibiting action led to an enhancement of enzyme activity. The substance to be tested was always mixed with the enzyme in the Thunberg tube, and the reaction was started when temperature equilibration had occurred. Control measurements showed that the enhanced reduction of dye was not due to uncatalysed reactions with the added substance.

The possibility that the active centre contains a disulphide grouping was also considered. Five hours' incubation with 0·1M $NaHSO_3$ and subsequent dialysis, however, had no effect on enzyme activity.

IV. Discussion

The ease with which the enzyme may be separated from the crushed cells under conditions in which autolytic changes are minimum strongly suggests that the enzyme is not firmly bound to the cell wall, or to insoluble particles in the cytoplasm. Since crude enzyme exhibits little or no oxygen uptake, arsenite oxidation in the intact cell must involve a loose association between the enzyme and the cytochromes, or, alternatively, some intracellular carrier. Facts presented in a subsequent paper (Legge 1954) make the first hypothesis the more likely.

Table 2

SUBSTANCES WHICH FAILED TO INHIBIT, OR WHICH ACCELERATED, ARSENITE DEHYDROGENASE ACTIVITY OF CLARIFIED CELL JUICE

Enzyme	Substance	Molarity	Activity $\frac{t \text{ control}}{t \text{ addition}} \times 100$
*L**	Lewisite oxide†	1×10^{-3}	100
	Chloroacetophenone†	2×10^{-3}	100
*T**	*o*-Iodobenzoate‡	1×10^{-3}	100
19.x.48	Pyrophosphate§	2×10^{-2}	118
		1×10^{-1}	127
20.x.48	Pyrophosphate§	2×10^{-2}	114
		1×10^{-1}	138
20.x.48	Pyrophosphate‖	2×10^{-2}	95
		1×10^{-1}	87
19.x.48	Phenyl $HgNO_3$§	2×10^{-4}	106
		2×10^{-3}	158
19.x.48	NaF§	1×10^{-1}	116
20.x.48	NaF§	1×10^{-1}	120
20.x.48	Iodoacetate§	1×10^{-2}	189
19.x.48	Thiourea§	2×10^{-2}	195
20.x.48	Thiourea	2×10^{-2}	230
11.xi.48	$(NH_4)_2SO_4$	$0 \cdot 5 \times 10^{-1}$	167
		1×10^{-1}	201

* Arsenite 20μM, indophenol 1μM, volume 2·5 ml, phosphate buffer, pH 6·8.

† Incubated for 1 hr.

‡ Incubated for 1 hr, and dialysed for 16 hr.

§ pH 5·9.

‖ pH 7·0.

Some enzyme appears bound to particles sedimenting more slowly than the cytochrome-rich pellet. These may be the bacterial analogues of the mammalian microsomes (Schachman, Pardee, and Stanier 1952), and the small part of the enzyme attached to such particles may be the fraction which Brachet and Jeener (1944) suggested is still associated with the systems which synthesize it.

The secular variation in enzyme activity observed in the course of many preparations, which was traced to the emergence of a variant, could not be related to any alteration of conditions of culture. It was, in fact, observed that decreased production of arsenite dehydrogenase was associated with decreased production of cytochrome *a* (Legge 1954).

There was no good evidence for our initial hypothesis that $-SH$ groups might be associated with the activity of the enzyme. Considerable inhibition was observed at a 2×10^{-3}M concentration of copper ion, but since none of the more powerful $-SH$ reactants gave substantial inhibition, and some even led to activation, this result may be due to binding at some other site than $-SH$.

It is difficult to explain the activation observed with some substances. The compounds implicated are dissimilar in nature, including a number of anions as well as thiourea, a neutral substance. With the exception of phenylmercurinitrate, the substances were present in relatively high concentration. Possibly in some instances the activation may have been due to inactivation of inhibiting impurities present in the crude cell juice; that these occur is suggested by the observation that when enzyme solutions were left at room temperature, a precipitate tended to form, and that the activity then increased.

V. Acknowledgment

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BACTERIAL OXIDATION OF ARSENITE

IV. SOME PROPERTIES OF THE BACTERIAL CYTOCHROMES

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Summary

Cytochromes *b* and *c* were observed in all substrains of *Pseudomonas arsenoxydans-quinque* examined; in some substrains, however, cytochrome *a* was not visible. Such strains poor in cytochrome *a* were less efficient arsenite oxidizers than those in which this cytochrome component was detectable.

The cytochromes were associated with the insoluble fraction obtained after grinding the packed cells with powdered glass, only traces of cytochrome *c* being found in the soluble fraction.

A significant rate of arsenite oxidation in systems reconstructed from dehydrogenase and oxidase was observed either when both fractions were present in relatively high concentration (about one-seventh that within the cell) or when an artificial carrier (2,6-dichlorophenol indophenol) was present.

Mammalian cytochrome *c* proved ineffective as a carrier. No evidence could be obtained that any natural carrier was present, and calculations indicate that diffusion of the arsenite dehydrogenase-arsenite complex could account for the oxygen uptake found in intact cells without any more readily diffusible electron carrier.

I. Introduction

A previous paper (Legge and Turner 1954) described some of the properties of the crude arsenite dehydrogenase which can be extracted from strains of *Pseudomonas arsenoxydans-quinque* grown in the presence of arsenite. The present paper describes some properties of the bacterial cytochromes in intact cells or in the sedimentable fractions obtained during the purification of the oxidase.

The observations have thrown some light on the extent of the changes observed during the variation in characteristics of the strain, reported in the above paper, but do not explain the altered sensitivity to carbon monoxide referred to in an earlier paper (Turner and Legge 1954).

II. Methods

General details for cultivation and harvesting of arsenite-oxidizing pseudomonads are given in earlier papers in this series.

The crude cytochrome oxidase fractions used in the present paper were obtained from the same batches of harvested cells used by Legge and Turner

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(1954) for the preparation of crude arsenite dehydrogenase. Fraction P_1 consisted largely of easily sedimentable cell debris (3500 r.p.m. for 30 min in the Wifug angle centrifuge); fraction S_1P, a mitochondria-like fraction, was thrown down as a reddish translucent pellet when the supernatant from P_1 was spun in a high-speed centrifuge (60 min at an average of 19,000*g*).

Spectroscopic observations were made with a Hartridge (Beck) or a calibrated small dispersion microspectroscope. Rate of reduction of cytochrome *c* was measured in a DU model Beckman spectrophotometer. Oxygen uptakes were measured by conventional manometric methods.

III. Results

(*a*) *Spectroscopic Observations*

Cytochromes *b* and *c* were invariably present when cells were examined after reduction with buffered arsenite. The *c* component was generally much stronger than the *b*, the α band of the *c* lying at 552 mμ.* Direct comparison left no doubt that the α band of bacterial cytochrome *c* lies slightly to the red side of that of cytochrome *c* of bakers' yeast or ox heart. Bacterial cytochrome *b* lay at about 560 mμ, but accurate measurement was not possible. Where cytochrome *a* was present, the α band lay at 600-603 mμ. It was not possible to observe any splitting of this band in the presence of CO and arsenite, even when the specimens were examined after freezing in liquid air, nor was any change observed with cyanide. Cytochrome a_2 was not seen. In the presence of pyridine and $Na_2S_2O_4$, the band of the pyridine haemochrome of cytochrome *a* was observed at 589 mμ.

If small amounts of arsenite were used, the cells could readily be oxidized on shaking with air. In agreement with the earlier deductions from the redox potential of the arsenite-arsenate system (Turner 1949), the addition of arsenite to oxygenated cells led to the appearance of the *c* band, followed by *b* and *a*. In the presence of malate, which is readily oxidized by the organisms, reduced *b* appears before *c*.

Spectroscopic examination of fractions *P* and S_1P, after fragmentation of the cells, added little to the above, although in some cases the S_1P fraction was richer in cytochromes than the *P* fraction. $Na_2S_2O_4$ produced immediate reduction, arsenite and its dehydrogenase a somewhat slower reduction. Attempts to observe preferential reduction of cytochrome *a* by leuco-indophenol failed.

The crude dehydrogenase solution clarified by high-speed centrifugation was greyish brown in colour and contained traces of cytochrome *c*.

The most interesting feature of the spectroscopic examination lay in the difference between the cytochromes in cells from strains with high or low arsenite-oxidizing capacity. The observations are summarized in Table 1.

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* We have referred throughout this series of papers to the cytochrome component absorbing maximally at 552 mμ as cytochrome *c*. Chin (1952) and Ryley (1952) refer to a component in the cytochromes of *Aerobacter* and *Tetrahymena pyriformis*, respectively, absorbing at 551-553 mμ, as cytochrome *e*, and restrict cytochrome *c* to the component absorbing at 550 mμ.

Stored cells (*R* to *X* inclusive), which were cultivated during the period of lowered arsenite dehydrogenase activity, were pink in colour, and contained only traces of cytochrome *a*. When substrain 17*L*2 was isolated from the cultures at this stage, it showed the original arsenite dehydrogenase activity, was brown in colour, and contained considerable amounts of cytochrome *a*.

Table 1

VARIATIONS OF CYTOCHROMES IN DIFFERENT BATCHES OF *PSEUDOMONAS ARSENOXIDANS-QUINQUE*

Source	Arsenite-oxidizing Activity	Cytochromes*	Colour of Preparation
Stored cells			
F	7700†	*c*, *b*, *a*	Brown
R	2200†	*c*, *b*, *a* absent	Extremely pink
S	2200†	*c*, *b*, trace of *a*	
T	3500†	*c*, *b*, trace of *a*	
V	2800†	*c*, *b*, *a* absent	
X	3900†	*c*, *b*, trace of *a*	
Z (substrain *L*2)	14,100†	*c*, *b*, *a*	Brown
AA	16,600†	*c*, *b*, *a*	
AB	14,200†	*c*, *b*, *a*	
AD	—	*c*, *b*, *a*	
Fresh cultures, May 1950			
Substrain *L*2, KA3	Moderately active	*c*, *b*, *a*	Brown
Substrain *L*2, YA3	Moderately active		
Substrain *L*2, K (no arsenite)	Inactive		
Substrain *X*, KA3	Poorly active	*c*, *b*, trace of *a*‡	Very pink
Substrain *X*, YA3	Poorly active	*c*, *b*, *a* absent‡	Very pink
Substrain *FB*, YA3§	Poorly active	*c*, *b*, *a* absent	
Fresh cultures, June 1950			
Substrain *L*2, KA3	180 μM As/mg N/hr‖	*c*, *b*, *a*	Brown
Substrain *X*, KA3	166 μM As/mg N/hr‖	*c*, *b*, *a*	Brown

* Cells or oxidase fraction observed in 1-2 mm deep paste in reduced state at room temperature, and immediately after being cooled to the temperature of liquid air.

† Measured anaerobically (cf. Legge and Turner 1954).

‡ These cells oxidized only slowly on shaking with air.

§ A large-celled variant isolated at the same time as substrains *L*2 and *S*.

‖ Manometric measurement in air.

The other substrain, 17*X*, isolated at the same time and thought to be the main organism, gave harvests of cells low in cytochrome *a*, and with low arsenite-oxidizing activity, in May 1950; but after further subculturing it regained its high activity, and became indistinguishable spectroscopically from 17*L*2, although still distinguishable from this substrain by colonial habit.

(*b*) *Specificity of Bacterial Cytochromes*

The work of Keilin and Hartree (1930) and of Slater (1949) provides the basis for tests of the donor specificity of cytochromes. The bacterial oxidases, however, differed from the mammalian in a number of ways.

Although cytochrome *c* in intact, adapted cells is readily reduced by arsenite, only traces appear to be liberated into solution when cells are disrupted. Nor could it be prepared from the crude bacterial oxidase; various treatments, including trichloracetic acid, strong salt solutions, and bile salts, were invariably unsuccessful in bringing any of the cytochrome *c* into solution.

TABLE 2

SUBSTRATE SPECIFICITY OF DIFFERENT SAMPLES OF STORED OXIDASE

Oxidase	Age (days)	Oxygen Uptake (μl O_2/mg N/hr†) on the Following Substrates*				Cytochrome *a*
		aa+*c*	*ppd*	*ppd*+*c*	*aa*+*ip*	
Fresh, partially purified ox heart	2	3150	2620	2820	3020	Present
AC‡	30	42	910	1060	1740	Not observed
AD	25	160	1080	—	3060	Present
AE	25	304	330	—	4060	Absent
R§	120	100	320	—	3500	Absent
S	112	220	560	—	4250	Trace

* *aa* (ascorbic acid 17×10^{-3}M), *c* (cytochrome *c* $0 \cdot 9 \times 10^{-4}$M), *ppd* (*p*-phenylene diamine 50×10^{-3}M), *ip* (2, 6-dichlorophenol indophenol $0 \cdot 6 \times 10^{-3}$M).

† Measurements in phosphate buffer, pH 6·8, 0·07M, 38°C, the gas phase was air.

‡ Fifth to seventh cultures respectively after isolation of substrain *L*2.

§ Oxidase at lowest point of arsenite dehydrogenase production.

Since preparations of cytochrome *c* from such widely different sources as wheat germ and mammalian heart have not been shown to differ markedly in specificity, attempts were made to use ox and horse-heart cytochrome *c* with the bacterial oxidase. The added carrier was found to have little or no effect on the rate of oxidation of *p*-phenylene diamine (cf. Table 2). The oxidase, however, was unsuccessful in bringing about any oxidation of ascorbic acid without added mammalian cytochrome *c*. In regard to the accessibility of the bound cytochrome *c* to those two donors, the bacterial oxidase resembles that of mammalian tissues. In investigations of the variation of the oxidase fraction in the different substrains, mammalian cytochrome *c* was therefore added when ascorbic acid was used, whereas *p*-phenylene diamine was used without added carrier.

In view of the ability of arsenite dehydrogenase to reduce 2,6-dichlorophenol indophenol, and of cytochrome oxidase to oxidize indophenols (Keilin 1925), a donor system comprising ascorbic acid and 2,6-dichlorophenol indophenol was also tested on mammalian and bacterial oxidases. Table 2 assembles results with these systems obtained in the course of analysis of stored oxidase preparations.

Comparison between the mammalian oxidase and the bacterial oxidases on the basis of their ability to oxidize leuco-2,6-dichlorophenol indophenol is of some interest. While the fresh mammalian specimen gives approximately the same activity whichever hydrogen donor system is used, the bacterial oxidase shows a far greater activity with the leuco dye as substrate, independent of the presence or absence of cytochrome *a*. Since the high redox potential of the indophenol-leuco-indophenol system makes it extremely unlikely that it is oxidized by enzymes other than those with redox potentials of cytochrome *a* level or above, such an oxidase, as yet undetected spectroscopically in these preparations, may be present.

Data obtained with oxidase from freshly harvested cells, prepared by grinding once-washed cells with powdered glass, are set out in Table 3.

TABLE 3

SUBSTRATE SPECIFICITY OF OXIDASE FROM FRESHLY HARVESTED CELLS

Oxidase from Substrain‡	Medium	Oxygen Uptake (μl O_2/mg N/hr†) on Substrates*		Intact Cells Plus Arsenite
		aa+*c*	*ppd*	
F_B	YA3	260	620	130
17*X*	K(no As^{--})	880	1480	59
17*X*	KA3	970	890	234
17*L*2	K(no As^{--})	880	2340	0
17*L*2	KA3	450	1980	370
17*L*2	YA3	170	1680	590

* *aa* (ascorbic acid 17×10^{-3}M), *c* (cytochrome *c* $0{\cdot}9\times10^{-4}$M), *ppd* (*p*-phenylene diamine 50×10^{-3}M).

† Measurements in phosphate buffer pH 6·8, 0·07M, 38°C, the gas phase was air. Uptake corrected for autoxidation of hydrogen donors.

‡ Cultivated May 1950, same samples as are referred to in Table 1.

The results in Table 3 indicate that the $Q_{O_{2ppd}}/Q_{O_{2aa+c}}$ ranged from 2·7 to 9·7 in the active 17*L*2 substrain, and from 0·9 to 2·4 in substrains 17*X* and 17*FB*, which were less effective arsenite oxidizers. The values of the arsenite oxidation by intact cells, however, given in the last column of the table show little correlation with the oxidase assays.

The activities with *p*-phenylene diamine and ascorbic acid-cytochrome *c* as substrates seem somewhat higher than those recorded with aged oxidase in Table 2, suggesting that there is an increasing impairment of function with age.

Ranking the $Q_{O_{2}ppd}$ (independently of substrain or culture medium) of those oxidase fractions for which spectroscopic observations on the presence of cytochrome *a* are available gives the arrangement in Table 4.

TABLE 4

ASSOCIATION BETWEEN CYTOCHROME *a* AND RATE OF OXIDATION OF *p*-PHENYLENE DIAMINE

μl O_2/mg N/hr	Cytochrome *a*
320	—
330	—
560	±
620	—
890	±
1080	+
1680	+
1980	+
2340	+

—, Not visible; ±, visible with difficulty in thickest parts; +, readily detectable.

This is in accord with the hypothesis that the activity against *p*-phenylene diamine is proportional to the cytochrome *a* content. The $Q_{O_{2}aa+c}$ shows no such correlation with the spectroscopic appearance of the preparations.

(*c*) *Reconstructed Oxidase Systems with Carriers*

No oxygen uptake was observed in preliminary experiments in which arsenite, arsenite dehydrogenase, and bacterial oxidase were shaken with air.

Replacement of phosphate by veronal buffer, or inclusion of calcium or magnesium, or both, did not lead to any increase in activity of the reconstructed system. No activation was found when extracts from boiled pseudomonads, yeast, guinea pig heart, or liver were used, or when mammalian cytochrome *c* was added. Since the bacterial oxidase is able to oxidize mammalian cytochrome *c*, and since arsenite is able to reduce the bacterial cytochrome *c* in the intact cell, the above result must be due to the slow rate of reduction of mammalian cytochrome *c* by the dehydrogenase.

This is borne out by a comparison between the rate at which the dehydrogenase reduces ferricyanide, measured manometrically in bicarbonate buffer, and the rate at which it reduces ferricytochrome at the same pH, measured spectrophotometrically. Ferricyanide (2×10^{-2}M) is reduced 10 times faster than $0{\cdot}6 \times 10^{-3}$M cytochrome *c*, and about 100 times faster than $0{\cdot}9 \times 10^{-5}$M cytochrome *c*.

Since arsenite readily reduces 2,6-dichlorophenol indophenol in the presence of the dehydrogenase, and since the oxidase fraction is able to oxidize an indophenol-ascorbic acid hydrogen donor system extremely rapidly, the dye may be used as a carrier between the arsenite dehydrogenase and the oxidase. Such an experiment is set out in Table 5.

With indophenol it is thus possible to use mammalian cytochrome oxidase to oxidize arsenite in the presence of the bacterial dehydrogenase.

Since the data in Table 5 show that oxidation proceeds rapidly in a dilute system with an artificial carrier, further attempts were made to prepare cell-free suspensions under conditions which would favour the preservation of any labile natural carrier, or which would reduce to a minimum changes in the cytochromes which might render them inaccessible to the dehydrogenase systems.

Table 5

RECONSTRUCTED ARSENITE-OXIDIZING SYSTEM WITH 2,6-DICHLOROPHENOL INDOPHENOL AS HYDROGEN CARRIER

Ox	*d*	As^{---}	*ip*	*ppd*	Fe(CN)$_6^{---}$	Oxidation (μ-equiv./hr)
−	+	+	−	−	+	2·5
+	−	−	−	+	−	7·3
+	+	+	−	−	−	0
+	+	+	+	−	−	5·9

Ox = oxidase Zp, 0·2 ml; *d* = arsenite dehydrogenase Zs, 0·025 ml; As^{---} = 0·05M arsenite; *ip* = 2,6-dichlorophenol indophenol, 1×10^{-3}M; *ppd* = *p*-phenylene diamine, 5×10^{-2}M; phosphate 0·07M; pH 6·8; 38°C; air in gas phase.

$K_3Fe(CN)_6 = 2 \times 10^{-2}$M; HCO_3^- buffer; N_2/CO_2 gas phase; 2·3 ml final volume.

The preparation of oxidase and dehydrogenase fractions from the bacteria generally involved some hours of manipulation at room temperature, although they were ground and subsequently stored at 0°C. Since these conditions might possibly have lead to enzymic destruction of part of the oxidase, bacteria were hand-ground with glass at 0°C; the debris was suspended and immediately transferred to manometric vessels, and arsenite oxidation was measured as soon as temperature equilibration had been reached. The rate of oxidation was found to be only 1/600 that of the intact cells. The results were the same when the cells were ground in the presence of 0·02M nicotinamide.

(*d*) *Reconstruction of Concentrated Systems Without Carriers*

While not providing unequivocal grounds for rejecting the hypothesis of exceptionally labile carriers, or uncontrolled alteration of the cytochromes, the above results suggest consideration of alternative hypotheses, such as the possibility that oxidation in the intact cell involves the diffusion of dehydrogenase-arsenite complex to the cytochromes, and that the failure to find oxidation in disrupted cell suspensions is due primarily to dilution effects.

This could be tested by measuring the oxygen uptake in extremely concentrated systems. Oxidase and dehydrogenase fractions from cultivation *AC* of substrain 17*L*2 were used after 14 days storage at 0°C. Both fractions were dialysed for 24 hr against water at 0°C in order to remove traces of metabolites or diffusible carriers. The activity per mg N of the oxidase was unaltered by this treatment, that of the dehydrogenase going up somewhat due to the loss of some low-molecular-weight nitrogenous compounds. After dialysis the dehydrogenase fraction represented a dilution about five times the volume of the original cells, whereas the oxidase fraction was diluted after dialysis to a pipettable consistency. A mixture of the two, in the volume ratio of 0·4 oxidase to 0·6 dehydrogenase, was then introduced undiluted, and diluted 2-, 4-, 8-, and 16-fold into manometric vessels. Strong phosphate buffer to give a final concentration of 0·07M, pH 6·8, was added, and arsenite was placed in the side-arms. To the vessel containing the 16-fold dilutions was added 10^{-3}M 2,6-dichlorophenol indophenol. Additional samples of 16-fold diluted solutions were assayed for dehydrogenase activity with ferricyanide, and for oxidase activity with indophenol and ascorbic acid. After 30 min equilibration to estimate blank respiration, arsenite was tipped in, and the measurements were continued for 10 hr, in the 4- and 8-fold dilutions. The uptakes remained perfectly linear over this period, indicating that growth of any organisms left after the crushing could be neglected. The results are set out in Table 6.

Table 6

RECONSTRUCTED ARSENITE-OXIDIZING SYSTEM WITHOUT ADDED HYDROGEN CARRIER

Arsenite Oxidation

Dehydrogenase : oxidase = 6/4	Rate (μl O_2/hr)	Enzyme N/Vessel (mg)	μl O_2/mg N/hr
Undiluted	273	6·68	38
Twofold dilution	86	3·54	24
Fourfold dilution	26	1·82	14
Eightfold dilution	12	0·99	11
16-fold dilution (indophenol as carrier)	89	0·50	178
16-fold dilution (ferricyanide as acceptor)	52*	0·50	104
16-fold dilution (indophenol and ascorbate as donors)	350	0·50	700

* Recalculated from CO_2 output.

The above figures are not corrected for the blank oxygen uptake. This presents some difficulty: from a knowledge of the total uptake to be expected from the amount of added arsenite, it can be calculated that the oxygen uptake due to hydrogen donors other than arsenite is decreased from 11 μl O_2/mg N/hr in its presence. The uptake observed in the 16-fold diluted vessel in the presence of indophenol indicates the inefficiency of oxidation in the unsupplemented vessels. Owing to an oversight, control measurements of the arsenite-oxidizing capacity of the separate fractions were omitted. However, if the uptake were due to a small amount of "active" dehydrogenase-oxidase complex, dissociable with difficulty, in either fraction, the activity should be independent of the dilution, and should not go up with increasing concentration.

The oxygen uptake with indophenol-ascorbic acid, recorded in the last column of Table 6, indicates that some component of the oxidase capable of oxidation at appropriate redox level is present in great excess at all dilutions.

The nitrogen content of the solutions containing the undiluted enzymes corresponds approximately to a seven-fold dilution of bacterial cell contents. If the reconstructed system differs from that within the intact cell only by being more dilute, one would arrive at a figure of 1860 μl O_2/mg N/hr ($7^2 \times 38$ μl O_2/mg N/hr) for the uptake of intact cells on the assumption of an approximate proportionality between activity and the square of reactant concentration (cf. Table 6). This should be compared with the observed figure of 810 μl O_2/mg N/hr. When the treatment to which the *AC* cells were subjected during fractionation is recalled, as well as the likelihood that much of the oxidase may be enveloped in structural debris, the calculated activity might well have been expected to be less than the observed activity. The fact that the latter is smaller could be due to higher intracellular viscosity or to non-uniform distribution of oxidase. When these considerations, for which adequate quantitative correction cannot be made, are taken into account, the knowledge that the observed figure disagrees with that calculated by a factor of only two is not unsatisfactory.

IV. DISCUSSION

If there is no diffusible electron carrier between arsenite dehydrogenase and oxidase, it is of interest to calculate whether diffusion of the activated arsenite dehydrogenase within the cell is sufficient to account for the observed oxygen uptake in the presence of arsenite.

In view of the simplifying assumptions necessary, such a calculation can only be expected to establish limits within which diffusion between large molecules would not be the limiting factor. Arsenite oxidation at rates of 100μM/mg N/hr in the presence of air may be achieved with present strains. The oxidase may be considered saturated at this oxygen pressure, because little increase in oxidation rate is observed in 100 per cent. O_2, and at 4 per cent. O_2 the rate only drops to 60 per cent. of that in air. Data presented earlier (Turner and Legge 1954) show that arsenite is oxidized to completion without much falling off in rate, even at substrate concentrations far below the optimum. This suggests that the permeability of the cell wall to arsenite is not a limiting factor under conditions in which uptakes of 100μM/mg N/hr were recorded.

Let us assume that the oxidase is evenly distributed throughout the cell, and is saturated with oxygen. Further, let as assume that the limiting factor within the cell is the diffusion of the activated arsenite-dehydrogenase complex to the oxidase.

The appropriate diffusion equations, which set limits to the radii of cylindrical and spherical organisms beyond which a diffusible compound being consumed uniformly throughout the mass of the organism does not reach the central region, are

$$r = \sqrt{4c\,D/A}$$

and

$$r = \sqrt{6c\,D/A},$$

respectively, where r is the radius, c is the concentration of metabolite, A is the rate at which the metabolite is being consumed, and D is the diffusion constant of the metabolite.

Since the arsenite-dehydrogenase complex exists only within the cell, c refers to the concentration at the outermost region of the cytoplasm. It is assumed that $D = 7 \times 10^{-7}$ cm^2 sec^{-1} for the arsenite-dehydrogenase complex, this being of the order for spherical proteins of molecular weight 40-50,000.

The organism is a rod, 2-3 μ long, and about 1 μ diameter, and its critical radius would probably be better described by an equation

$$r = \sqrt{5c\,D/A},$$

the constant being arbitrarily taken as lying between that for a rod, 4, and for a sphere, 6.

The oxygen uptake is equivalent to 100μM/mg N/hr. Converting this to moles cm^{-3} sec^{-1}, and assuming the nitrogen content to be 2 per cent. of the wet weight, a value is reached for $A = 5{\cdot}5 \times 10^{-7}$ moles cm^{-3} sec^{-1}. Inserting this value for A, 7×10^{-7} cm^2 sec^{-1} for D, and $0{\cdot}5 \times 10^{-4}$ cm for r, in the converted equation

$$c = \frac{r^2 A}{5D},$$

we obtain

$$c = \frac{2{\cdot}5 \times 10^{-9} \times 5{\cdot}5 \times 10^{-7}}{5 \times 7 \times 10^{-7}} \text{ moles cm}^{-3}$$
$$= 3{\cdot}9 \times 10^{-10} \text{ moles cm}^{-3},$$

or a concentration of activated enzyme-substrate complex of $3{\cdot}9 \times 10^{-7}$M.

The wet organism contains about 13 per cent. total protein, and 5 per cent. soluble protein. The assumption of a mean molecular weight of 48,000 for the latter leads to a figure for the molarity of $1{\cdot}0 \times 10^{-3}$, in which case the enzyme would represent a fraction of only $3{\cdot}9 \times 10^{-4}$ of the total soluble protein, or $3{\cdot}9 \times 10^{-2}$ per cent.

Few readily accessible data are available on the enzyme content of microorganisms expressed in units of mass rather than in units of activity. The above estimate that the arsenite dehydrogenase content of *Pseudomonas arsenoxydansquinque* is $3{\cdot}9 \times 10^{-2}$ per cent. of the soluble protein would appear reasonable. It is certainly far lower than the catalase content of *Micrococcus lysodeicticus* found by Herbert and Pinsent (1948); this was 1-2 per cent. of the dry weight, or of the order of 2-4 per cent. total protein or 4-8 per cent. soluble protein.

Certain of the assumptions made in deriving the estimate may now be reconsidered. It is obvious that it makes little difference whether diffusion formulae appropriate for cylinder or sphere are taken as the basis of the calculation. If the enzyme is assumed to be very much larger and to have a diffusion constant one-tenth that of a spherical protein of molecular weight 48,000, it would still represent only 0·39 per cent. of the total soluble cell protein.

The oxidase was assumed to be evenly distributed throughout the body of the cell. The fact that little may be separated from the insoluble fraction of crushed cells, a great part of which may be cell wall, suggests that the oxidase may be found in higher concentration at the periphery of the cell. This situation would require a different diffusion equation from the one used, but it is unlikely that this would lead to impossibly high values for the arsenite dehydrogenase content. If the dehydrogenase was loosely attached to oxidase within the cell, a far lower concentration would be needed to give the rates of arsenite oxidation found, irrespective of whether the oxidase is assumed to be distributed peripherally or evenly within the cell.

The calculation has been made on an arsenite oxidation rate of 100μM/mg N/hr found for the strains under present cultivation. The calculated enzyme content is so low that increased amounts could well be present in strains with greater arsenite-oxidizing ability.

The conclusion from diffusion theory therefore does not conflict with that reached from experiments on reconstructed systems, namely, that the observed oxygen uptakes in intact cells may be accounted for without postulating a diffusible carrier between dehydrogenase and oxidase.

V. ACKNOWLEDGMENTS

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THE MECHANISM OF FOOD DISTRIBUTION TO MIDGUT OR DIVERTICULA IN THE MOSQUITO

By M. F. Day*

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Summary

An examination has been made of factors involved in the despatch of sugars to the diverticulum and blood to the midgut in the mosquito. It is suggested that sense organs observed in the buccal cavity of the mosquito detect the presence of some sugars and components of blood. The resulting nervous impulse is probably transmitted via the stomatogastric nervous system to cause the contraction of sphincter muscles of the diverticula or the proventriculus. The significance of this switching mechanism in mosquito feeding is discussed.

I. Introduction

A knowledge of how mosquitoes transmit virus diseases requires an understanding of their processes of ingestion and digestion. One of the peculiarities of mosquito digestion that has been known for a long time is that, whereas whole blood passes directly to the midgut, solutions of sugars generally pass to the diverticula, rather than to the midgut. The problem of the distribution of liquids to these regions of the mosquito gut has recently been studied by Trembley (1952). Her main conclusions, incorporating some of those of earlier workers whom she quotes, may be summarized as follows:

(1) Blood, irrespective of the manner in which it is fed, goes primarily to the midgut. In some species practically none reaches the diverticula, in others a considerable amount of blood may go to them.

(2) Solutions of sugars (glucose or honey) go primarily to the diverticula, mainly the ventral diverticulum. Traces may go to the midgut at once, but normally the contents of the ventral diverticulum are later passed to the midgut, a small amount at a time.

The establishment of these facts does not answer the question of how various liquids reach the respective regions of the gut and, in fact, no comprehensive suggestion has been advanced that does provide an explanation of what Fisk (1950) has called the "switching mechanism." Boissezon's (1930) suggestion that the diameter of the ducts to the diverticula was not great enough to permit the passage of erythrocytes does not account for all of the observations, and Marshall and Staley's (1932) conclusions concerning the action of sphincters at the openings of the diverticula offer no indication of how these sphincters are activated. Fisk (1950) performed some experiments to determine what properties of blood enabled it to reach the midgut, but stated that his results did not solve the problem.

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Consideration of the conclusions of Trembley referred to above and of the anatomical relationships of the gut of the Diptera suggested that sense organs located in or near the buccal cavity may be stimulated by components of the food, resulting in the transmission of motor impulses to the sphincters of the diverticula or the proventriculus, or both.

Attempts to obtain data to evaluate this suggestion are reported in this paper. The results permit the formulation of a hypothesis that appears to provide an explanation of the "switching mechanism."

II. Materials and Methods

Of the species of mosquitoes examined by Trembley (1952), *Aedes aegypti* (L.) appeared to exhibit the switching mechanism to a marked degree, and it was therefore suitable for this work.

As the method of feeding does not influence the destination of liquids ingested (Bishop and Gilchrist 1946; Trembley 1952), heparinized rabbit blood and blood and sugar mixtures were fed to starved *A. aegypti* from cotton wool or filter paper soaked in the fluid. Liquids other than blood were generally coloured with carmine. Mosquitoes, kept overnight in small glass vials covered with mosquito netting, could frequently be stimulated to ingest blood and sugar mixtures by having them briefly probe the skin of the forearm and immediately removing them to small dishes (containing the cotton wool and substances to be tested) standing on a thermostatically controlled warm plate. Difficulty was experienced in getting numbers of mosquitoes to accept some of the mixtures, particularly those lacking erythrocytes. Larger numbers of fed mosquitoes of both sexes were obtained in several instances by collecting insects as they fed from saturated filter papers in dishes containing the blood-sugar mixtures. Dissections were performed in a drop of *Drosophila* Ringer.

Ten-micron serial sections of mosquitoes stained with Mallory's triple connective stain or by Bodian's protargol method were used to study the sense organs. The anatomy, innervation, and musculature of the gut were studied also in whole mounts of the gut after staining with methylene blue by the method of Kuwana (1935). Phase-contrast microscopy, using a Leitz Heine condenser, was used to study the crop musculature and innervation.

III. Feeding Experiments

Although several earlier authors have reported the destination of mixtures of blood and sugars in the mosquito gut, the data are inadequate for the present purposes. Experiments were therefore performed to determine what components of the blood or blood : sugar mixtures were stimulatory and what were the approximate thresholds for stimulation. The results (Table 1) demonstrate that, in agreement with the work of earlier authors, blood reaches the midgut whereas glucose solutions go mainly to the diverticula.* The addition of a small amount of blood to 10 per cent. glucose solution results in the mixture going partly to the midgut and partly to the diverticulum. Thus 1 part of blood to 20 of glucose was sufficient to be detected and to activate the switching mechanism. Over a wide range of concentrations of blood in mixtures of glucose solutions the mixture went both to the midgut and to the diverticulum.

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* Frequently liquids were found only in the ventral diverticulum, whereas the dorsal diverticula contained gas bubbles. Only the ventral diverticulum was considered in the results presented.

TABLE 1

DESTINATION OF MIXTURES OF 10 PER CENT. GLUCOSE AND WHOLE BLOOD WHEN INGESTED BY *AEDES AEGYPTI*

Composition of Liquid	Molar Concn. of Glucose	Number Engorged	Destination (%)	
			Diverticulum	Midgut
Blood	—	50	6*	100
Blood 3 ml; glucose 1 ml	0·08	25	16	92
Blood 2 ml; glucose 1 ml	0·13	20	40	70
Blood 1 ml; glucose 1 ml	0·25	30	73	80
Blood 0·5 ml; glucose 1 ml	0·30	30	83	86
Blood 0·25 ml; glucose 1 ml	—	20	80	90
Blood 0·1 ml; glucose 1 ml	0·46	30	100	7
Blood 0·05 ml; glucose 1 ml	0·50	25	100	8
Glucose 1 ml	—	50	100	10

* Trace only. (Note that particularly at intermediate concentrations the ingested liquid frequently goes to both midgut and diverticulum in the same insect.)

If glucose is added to heparinized blood in sufficient concentration, the mixture again goes both to the midgut and diverticulum. The conclusion is inescapable that both whole blood and glucose are detected, and the relative concentration of the components of a mixture determines its destination. These results suggested the search for separate receptors to detect glucose and blood (see Section IV below).

The experiment was repeated replacing glucose by other sugars. Equal parts of 5 per cent. sucrose and heparinized blood went mainly to the diverticulum, indicating that sucrose was detected (Table 2). On the contrary, a mixture of 2 ml 10 per cent. lactose in 1 ml heparinized blood went entirely to the midgut, suggesting that lactose was unable to stimulate the receptors. Insufficient numbers of mosquitoes for a study of thresholds could be induced to accept blood mixed with arabinose, mannose, and raffinose, but all three could apparently be detected, for when these sugars were mixed with blood at a concentration of 0·2 g/ml the mixture went both to the diverticulum and to the midgut.

Experiments were next designed to determine what components of whole blood were responsible for the switching mechanism. Similar experiments performed by Fisk (1950) did not permit conclusions to be drawn from them, but Bishop and Gilchrist (1946) found that plasma, washed erythrocytes, and haemolysed blood when ingested went to the midgut and not to the diverticulum. These data and the results presented in Table 2 suggest that both plasma and erythrocytes are detected but the reaction to plasma is less marked than that to washed erythrocytes. Solutions of haemoglobin and albumin with glucose went mainly to the diverticulum, and were thus presumably not detected; however, these substances were ingested by too few mosquitoes for the results to be unequivocal.

TABLE 2

DESTINATION OF MIXTURES OF SUGARS AND BLOOD AND BLOOD CONSTITUENTS WHEN INGESTED BY *AEDES AEGYPTI*

Composition of Liquid	Number Fed	Destination (%)	
		Diverticulum	Midgut
Blood 5·0 ml; 3·5% sucrose 1·0 ml	20	0	100
Blood 0·3-0·2 ml; 3·5% sucrose 1·0 ml	20	100	100
Blood 1·0 ml; 5% sucrose 1·0 ml	20	100	10
Erythrocytes* 0·3 ml; 5% glucose 1·0 ml	20	50†	100
Erythrocytes* 0·1 ml; 5% glucose 1·0 ml	20	100	20‡
Plasma§ 1·0 ml; 5% glucose 1·0 ml	20	100	45
Plasma§ 1·0 ml; 5% sucrose 0·5 ml	20	80	30
Plasma	20	100	20

* Prepared by twice centrifuging heparinized rabbit blood and resuspending the sedimented cells in physiological saline to the original volume.

† In three of the 10 insects of the group, solution, but no erythrocytes were present in the diverticulum.

‡ Erythrocytes present in midgut only, none in diverticulum.

§ Supernatant from first centrifugation of * above.

When mixtures of washed erythrocytes and glucose were fed to mosquitoes, the mixtures went to the midgut, the diverticulum, or to both, depending upon the concentrations of the components. When the concentration of washed erythrocytes was low, however, a number of mosquitoes were found to have diverticula filled with a clear liquid, whereas the midguts contained liquid together with a few erythrocytes. The straining mechanism mentioned by Boissezon (1930) appears to be effective in these instances. The occurrence of erythrocytes in the diverticulum when blood : sugar mixtures in other concentrations are fed demonstrates, however, that this straining apparatus is not normally able to control the switching mechanism.

A group of spines was observed in the neck of the ventral diverticulum (Plate 1, Fig. 5). These do not seem to have been noticed by previous workers; they differ in shape and arrangement from those described in the pyloric region of the same species by Trembley (1951). Their presence would assist in keeping erythrocytes out of the diverticulum when the circular muscles at the neck were contracted, and they probably function in straining out the erythrocytes as described in the previous paragraph.

The response to washed erythrocytes suggested that the stimulus might be tactile rather than gustatory. An attempt to examine this possibility was made by feeding mosquitoes on washed red cell ghosts. Rabbit erythrocytes were haemolysed in distilled water, centrifuged, resuspended in distilled water, and centrifuged again. The resulting ghosts, pinkish in colour, were resuspended in a minimum of water and fed to 7-day-old, previously unfed mosquitoes. Five ingested these ghosts and in all the suspension went mainly to the midgut. Thus washed red cell membranes, though less readily accepted than erythrocytes, were able to activate the switching mechanism.

It was possible that residual haemoglobin may have been responsible for causing the membranes to be despatched to the midgut; it was therefore considered desirable to determine the destination of other particulate material after ingestion by the mosquito. Several such materials (water-insoluble dyes, starch grains) were not ingested. Finally, starved mosquitoes did ingest washed sarcosomes separated from the thoracic muscles of the blowfly *Lucilia cuprina* (Wied.). When suspended in distilled water, these approximated the diameter of mammalian erythrocytes. In 20 *A. aegypti* females that ingested this suspension, the material was found both in the midgut and the diverticulum. The supernatant from a centrifuged preparation went only to the diverticulum. It seems likely, therefore, that it was the particulate nature of the sarcosomes that caused some of the suspension to be despatched to the midgut.

In some experiments males were used in addition to females. The two sexes reacted similarly; blood went to the midgut and sugar to the diverticulum in the male in spite of the fact that males do not ingest blood under natural conditions.

In some early experiments it seemed possible that the age of the mosquitoes or their previous feeding were responsible for variations in their behaviour with respect to the destination of blood : sugar mixtures. A series of tests was therefore set up to determine whether these factors were contributing to the variability of behaviour. In one test comparisons were made between 2-day-old females previously unfed and 7-day-old females that had had access to sugar water for the first 6 days after emergence. There was no difference in the destination of blood : glucose mixtures in the two groups. In a second test no difference in the destination of blood : glucose mixtures was detected in a comparison between females that had had no previous feed and females that had had a single blood meal 7 days previously. Variability in destination occurred equally in all groups, and no method was developed which resulted in completely uniform behaviour of any groups of insects towards blood : glucose mixtures. There was much more marked uniformity, however, in response to sugar or to whole blood offered separately.

IV. Anatomical Considerations

The feeding experiments reported in Section III suggested that two types of sense organs might be necessary to detect both sugars and the components of blood. Although pharyngeal sense organs have been recorded in several insects (e.g. in larval *Drosophila* (Hertweck 1931), *Glossina* (Jobling 1933), and in *Panorpa* (Grell 1938)), no record of their occurrence in mosquitoes could be found. Examination of the stylets showed the absence of appropriate receptors in these chitinous structures (see also Robinson 1939). Serial sections of the head of *Aedes aegypti* revealed the presence of the dorsal and palatal papillar sense organs in the buccal cavity. These were first described by Sinton and Covell (1927), and by Barrand and Covell (1927), who showed that their form and arrangement had considerable taxonomic value. The location and arrangement of these sense organs in *Aedes aegypti* is shown in Figures 1 and 2. Sections showed that the sense organs were of four types, as illustrated in Plate 1, Figures 1-4; the three pairs of palatal organs were spines, whereas the three pairs of dorsal organs were hair-like receptors and there was a single pair of campaniform papillae. Similar structures were found in male *A. aegypti*. The cibarial sense organs described in adult *Drosophila* by Hertweck (1931) and Miller (1950) are probably homologous with these papillar sense organs of mosquitoes.

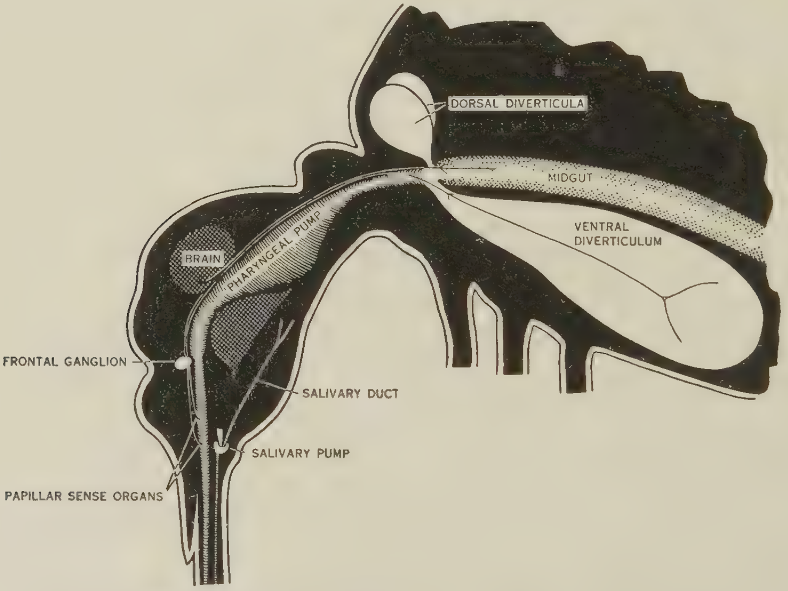


Fig. 1.—Diagrammatic median longitudinal section of anterior part of a mosquito, showing foregut and midgut and the stomodeal nervous system.

The precise function of the papillar sense organs cannot, of course, be certainly adduced from their structure; but the form of the palatal papillar organs is not inconsistent with the suggestion that they are chemoreceptors; the structure and position of the dorsal papillar receptors suggest that they may be capable of detecting the flow of erythrocytes and of the sarcosomes mentioned in the previous section. One of the four types of sense organ is apparently able to detect the presence of blood plasma.

The innervation of the seven pairs of sense organs could not be traced with certainty, but all received nerves from the frontal ganglion. (Miller (1950) believed also that the pharyngeal nerves that innervate the cibarial sense organ of *Drosophila* are homologous with the frontal ganglion connectives, and Wenk (1953) has shown that the labral nerves of the flea *Ctenocephalus* innervate cibarial sense organs.) It therefore seems likely that nerve impulses from the mosquito sense organs reach the stomatogastric nervous system. In all insects the stomatogastric nervous system innervates the foregut as well as the incretory organs of the head. This has been well shown in *Calliphora* (Graham-Smith 1934), and a similar arrangement has been revealed in favourable methylene blue preparations of *Aedes aegypti*. Orlov (1924) has shown that the frontal ganglion, of *Oryctes* larvae at least, contains sensory as well as motor nerve cells. The origin of these cells has never been elucidated. The experiments of Bolwig (1952) suggest that the frontal ganglion is not concerned with peristaltic movements in the fly gut and its function is not completely understood.

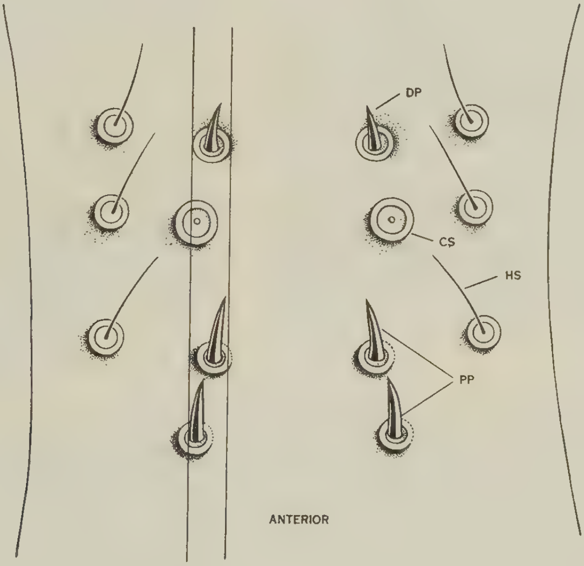


Fig. 2.—Diagram of number and arrangement of sense organs in the dorsal wall of the buccal cavity of *Aedes aegypti*. Palatal papillar sense organs (*PP*); campaniform sensillum (*CS*); dorsal papillar sense organ (*DP*), and hair-like sensilla (*HS*). The parallel lines indicate the plane of the section of Plate 1, Figure 4.

The innervation of the diverticula of *Aedes* is shown semi-diagrammatically in Figure 1. The arrangement of the musculature in an expanded ventral diverticulum and the relative size of the nerve and its method of branching are shown in Plate 1, Figure 6.

The origin of the nerves innervating the muscles of the diverticula is still not definitely known, but it seems most likely that they form part of the stomatogastric nervous system. It is not essential for the present purposes to know the precise neural pathway between the sensory receptors and the effector system. It is entirely possible that some central connections exist. However, the pathway through the frontal ganglion and the stomatogastric nervous system appears possible and is the simplest way of accomplishing the connections.

V. Discussion

The following hypothesis to explain the switching mechanism in the mosquito is suggested by the data presented above. Certain sugars stimulate the pit organs in the buccal cavity of the mosquito, causing impulses that result in the relaxation of the sphincters of the diverticula. Certain components of the blood of vertebrates stimulate the papillar sense organs, resulting in relaxation of the cardiac sphincters. Simultaneous stimulation of both groups of sense organs may result in relaxation of both series of sphincters. Both male and female mosquitoes react in the same manner to these ingested materials, even though the males never obtain a blood meal under natural conditions.

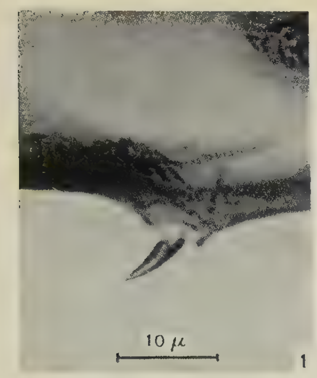
The thresholds of the pit organs to sucrose and glucose are somewhat high in comparison with tarsal and palpal receptors (see Dethier 1953) but these receptors would respond to the concentration of sugars normally found in nectars.

The function of the diverticula as containers of ingested sugar solutions does not, of course, preclude them from having additional functions. In some of the present experiments they were found to function also as "air separators" as previously shown by MacGregor (1930).

The question may be raised of the survival value of the switching mechanism in the mosquito and of the absence of such a mechanism in, for example, the blowfly, in which the necessary anatomical requirements would appear to be well developed. It is suggested that the answer is to be found in the differences in the feeding behaviour of the two Diptera. In the majority of species female mosquitoes require a blood meal in order to mature their eggs. The opportunity to bite a suitable vertebrate to provide the blood meal will come at irregular intervals and the insect must be capable of utilizing the opportunity when it is presented. Nectar is more generally available and is sufficient to keep the insect alive. But the ability to take a blood feed in spite of a recent nectar meal would seem to be of survival value, and this is what is accomplished by the "switching mechanism"; a crop containing some nectar does not preclude the taking of a blood meal should this become available.

Trembley (1952) has shown that the switching mechanism is not as well developed in some species of mosquitoes as it is in *A. aegypti*. It may be that the species of *Anopheles* and *Culex* that she studied, in which erythrocytes were often found in the diverticula as well as the midgut, have either less sensitive receptors in the buccal cavity, or a less well-developed sphincter musculature in the necks of the diverticula.

FOOD DISTRIBUTION IN THE MOSQUITO



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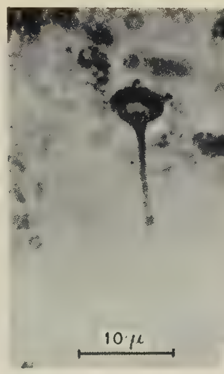

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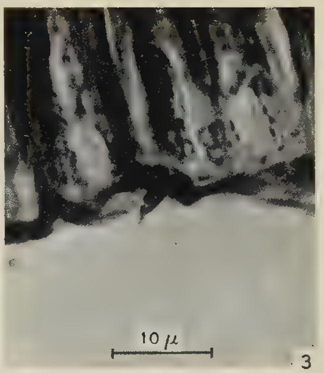

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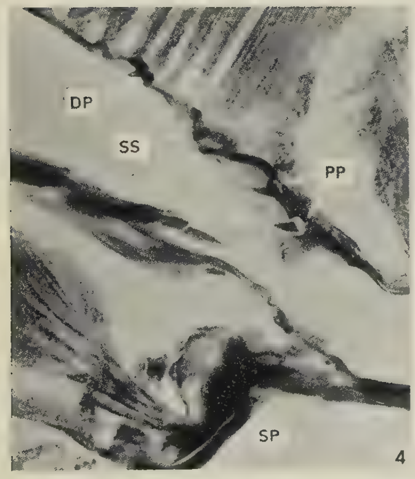

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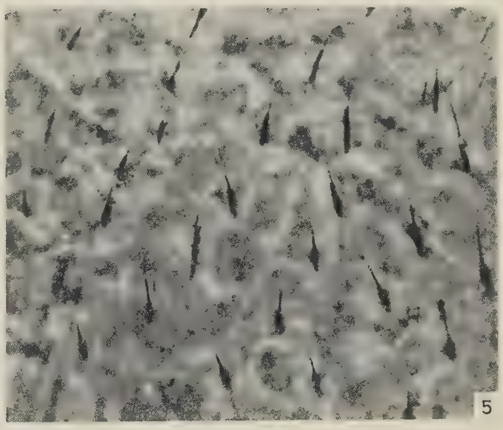

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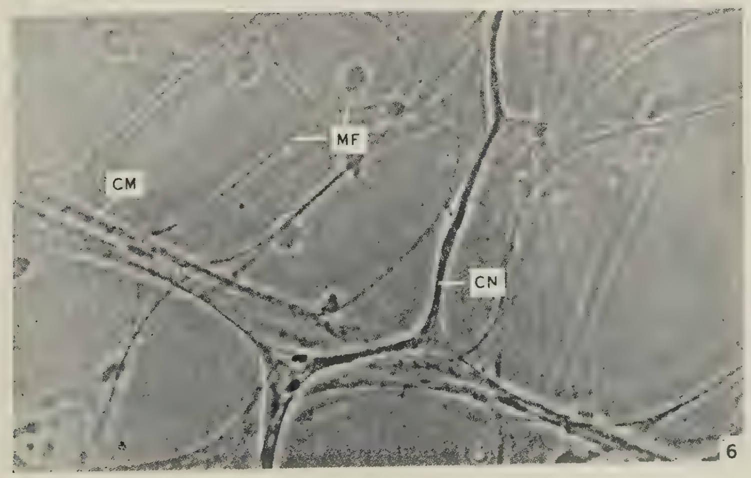

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VI. ACKNOWLEDGMENTS

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EXPLANATION OF PLATE 1

Photomicrographs of *Aedes aegypti*.

Fig. 1.—Longitudinal section of dorsal wall of buccal cavity showing structure of the palatal papillar sense organ projecting into the lumen. The spine is directed posteriorly.

Fig. 2.—The same, showing one of the six hair-like sensillae.

Fig. 3.—The same, showing structure of a dorsal papillar sense organ.

Fig. 4.—Longitudinal section of the buccal cavity, showing two palatal papillar sense organs (*PP*), a campaniform sensillum (*CS*), and a dorsal papillar sense organ (*DP*). The salivary pump (*SP*) is also shown. The anterior end of the insect is to the right of the illustration.

Fig. 5.—Whole mount of neck of ventral diverticulum, showing spines. These vary in length from about 15 to 30 μ. Phase contrast.

Fig. 6.—Whole mount of expanded crop showing arrangement of circular muscles (*CM*) and irregular network of fine muscle fibres (*MF*) connecting them and the crop nerve (*CN*), and its method of branching. The dark oval bodies are nuclei of the epithelium. Phase contrast.

REGENERATION AND THE MOULTING CYCLE IN *BLATTELLA GERMANICA* L.

III. SUCCESSIVE REGENERATION OF BOTH METATHORACIC LEGS

By A. F. O'Farrell* and A. Stock*

[*Manuscript received April* 2, 1954]

Summary

At 29°C, 70 per cent. relative humidity, removal of the left and right metathoracic legs in succession, at intervals of 1, 2, or 3 days, in first instar *Blattella germanica* of known ages, results in the appearance at the first moult of:

(*a*) Two asymmetrical papillae, the timing of the moult being undisturbed; or

(*b*) A regenerate on the left and a papilla on the right side, the time of moulting being determined by age at the *first* operation and independent of the second; or

(*c*) Two asymmetrical regenerates, the time of moulting being then determined by age at the *second* operation and independent of the first.

In (*b*) and (*c*) the rather constant size of regenerates from the first operation exceeds that of regenerates derived from either unilateral or simultaneous operations, while the smaller regenerates from the second operation in (*c*) show wide but erratic variation in size, not regularly related to the timing of the operations or the moult.

In about 2 per cent. of the material, the *second* operation *only* gives rise to a structure intermediate between a papilla and a complete regenerate. Such rare exceptions to the "all or nothing" principle can appear only when ecdysis occurs during the exceedingly rapid process of differentiation of the blastema into a regenerated leg. This proceeds proximodistally in these individuals, in contrast to animals producing a regenerate and a papilla at their first moult, where the rapid differentiation of the blastema contained within the papilla and its coxa occurs, early in the second instar, in the opposite (i.e. distoproximal) direction.

These results conform closely to predictions derived from the initial hypotheses put forward regarding the relationship between regeneration and the moulting cycle. New information on the regeneration process has also been obtained.

I. Introduction

Possible interrelations between regeneration and the endocrine balance controlling moulting in *Blattella germanica* have been discussed in earlier papers of this series (O'Farrell and Stock 1953; Stock and O'Farrell 1954). In these discussions it was suggested that regeneration, in certain conditions, produces a sort of physiological "putting back the clock," which involves a fresh start in the rhythm of the moulting cycle, dating from the time of removal of a metathoracic leg. Such a secondary moulting cycle, once established, should

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then be capable of modification, and even perhaps subjected to a further fresh start, by the onset of a second regeneration process appropriately timed. The postulated modification, however, would not be fully manifested unless the moulting cycle were relatively little influenced by the extra quantity of tissue to be produced in completing the regeneration of two appendages instead of only one. Since it has been shown (Stock and O'Farrell 1954) that simultaneous regeneration of both metathoracic legs during the first instar influences the first moult only very slightly more than unilateral regeneration of a single metathoracic leg, this condition appears to be fulfilled.

The present work, then, is an investigation of the nature of the secondary cycle set up by regeneration, and of the types of regenerate produced as a result of successive operations. It has been assumed that satisfactory evidence on these matters will be produced by examining the consequences of removing the two metathoracic legs in succession, at suitably varied intervals.

II. Material and Methods

The general cultural and experimental methods used were those described by O'Farrell and Stock (1953), but the animals were kept throughout in a constant-temperature room at 29°C, 70 per cent. relative humidity (R.H.) (± 0·5°C, and approx. 8 per cent. R.H.). Experimental material was obtained by subdivision of the batches used in work on simultaneous regeneration (Stock and O'Farrell 1954) so that the results of the two series of experiments would be directly comparable with one another and with the same population of controls.

Removal of one metathoracic leg at the proximal autotomy plane was carried out on animals of known age on each of the first 4 days of post-embryonic life, and followed at intervals of 1, 2, or 3 days by removal of the other metathoracic leg, in accordance with the schedules in Table 1. In the early stages, the first operation was performed alternately on the right and left side. This procedure was abandoned after results from about 300 experimental animals had confirmed the expectation that the overall nature of the regenerates and the timing of the first two moults were unaffected by the side on which the first operation was performed. Thereafter the standard practice adopted was to remove the left leg at the first and the right leg at the second operation.

Representative series of preparations of the metathorax, bearing regenerates produced from each combination of treatments, were fixed in alcoholic Bouin's fluid, stained in alcoholic light green, and mounted whole for detailed examination, measurement, and photography.

III. Experimental Results

(a) *Morphology of Regenerates*

The headings of columns 4-7 in Table 1 briefly describe the four types of combinations of regenerated structures observed at the first moult, after successive removal of the metathoracic legs in accordance with the nine operating schedules shown in columns 2 and 3 and allotted reference letters in column 1 of Table 1. These abbreviated descriptions and reference letters will be used throughout this paper.

TABLE 1

OPERATING SCHEDULES IN RELATION TO NUMBERS OF ANIMALS PRODUCING VARIOUS COMBINATIONS OF REGENERATES AT THE FIRST MOULT

Operating Schedule	Age (days) at		Numbers of Animals Producing				Total Numbers Surviving First Moult	
	First Operation	Second Operation	Two Papillae	Regenerate and		Two Regenerates	Experimentals	Controls
				Papilla	Intermediate			
A	0-1	2-3	0	0	0	24	24	24
B	0-1	3-4	0	33	2	58	93	90
C	1-2	2-3	0	0	0	85	85	105
D	1-2	3-4	0	21	9	113	143	144
E	1-2	4-5	0	73	2	25	100	99
F	2-3	3-4	19	3	3	108	133	149
G	2-3	4-5	70	6	5	81	162	172
*H**	2-3	5-6	11	47	1	18	77	94
J	3-4	4-5	125	0	0	28	153	137
Total numbers observed			225	183	22	540	970	1014

* The second operation in schedule *H* occurs after the majority of fast-growing animals have already moulted and have to be discarded, hence the very low proportion of "two papillae."

A detailed account of the four combinations of regenerates follows.

(i) *Two Papillae.*—This condition occurred only in association with operating schedules *F, G, H,* and *J*. Usually (Plate 1, Fig. 1) the side of the first operation (hereafter called "side I") showed a perfectly healed, smoothly rounded papilla, without trace of external differentiation or of clotted blood, replacing the original trochanter. On the side of the second operation ("side II") there was a blood-clotted, partly healed trochanter, showing neither the smooth rounding-off nor the associated reduction in size sometimes observed on side I. At the first moult, dedifferentiation of the coxal musculature and the beginning of blastema formation were often recognizable on side I, but not on side II (Plate 1, Fig. 1).

A less common form of "two papillae" occurred with a longer time lapse between the second operation and the first moult. Rounding-off, smoothness, reduction in size, and completeness of healing, denoted by the absence of any trace of clotted blood, were apparent in the trochanters of both sides (Plate 1, Fig. 2). They were usually asymmetrical, but the complexities of the healing process and subsequent events of dedifferentiation and blastema formation appeared to be such that the larger papilla was sometimes on side I and sometimes on side II, presumably according to the precise time relations between operation and moulting in the individual concerned. Internal changes visible at the first moult of such animals usually included some blastema formation, but internal asymmetry was not so clearly recognizable as in the material with a persistent blood-clot on side II.

Marked asymmetry in the size and differentiation of the blastemata on the two sides became apparent during the second instar, but tended to diminish towards the time of the second moult. At the second moult, two completely regenerated legs, with the usual tetramerous tarsi, appeared in place of the papillae. These regenerates were usually nearly symmetrical in size, and apparently completely so in their level of differentiation (Plate 1, Fig. 3).

Preparations of these papillae and of the regenerates which followed them were carefully compared with equivalent preparations from the experiments on simultaneous regeneration. Apart from the asymmetry of the two sides, the types and sizes of papillae resulting from the successive operations did not appear to differ from those produced after simultaneous operation. The regenerates appearing at the second moult showed a slight size asymmetry, usually detectable only by measurement, but otherwise appeared similar to those observed in similar circumstances in the work on simultaneous regeneration.

(ii) *Regenerate and Papilla.*—Occurring mainly in association with schedules *B*, *D*, *E*, and *H*, rarely with *F* and *G*, and never with *A*, *C*, or *D*, this condition was the least symmetrical observed. A large, highly differentiated complete regenerate with tetramerous tarsus appeared on side I, and a completely healed, smoothly rounded papilla, without external differentiation and showing no sign of clotted blood, replaced the trochanter on side II. This papilla seldom showed much size reduction by comparison with a normal trochanter, and occasionally even exceeded it in length; but such variations were sporadic and irregular, not being clearly related to the timing of operation or moulting, or both. At the first moult, the papilla usually showed considerable blastema development, extending into the distal musculature of the coxa (Plate 1, Fig. 4). The timing of the operations in relation to the moult had no recognizable or regular effect on the size or the differentiation of the regenerate on side I, which was rather consistently larger in size than comparable products of either unilateral or simultaneous regeneration.

Rapid differentiation of the blastema on side II was seen early in the second instar (Plate 2, Figs. 7 and 8), all essential features of a complete regenerate being apparently fully formed within 2-3 days after the first moult (Plate 2, Fig. 9). The numerous preparations studied suggested that the differentiation sequence was mainly distoproximal, with the tarsus being formed first and the femur last; but it is not yet clear to what extent departures from such a sequence may occur. Associated with differentiation was a certain amount of sculpturing of the cuticle of the papilla by the pressure of the growing blastema within. No evidence was obtained to suggest that *true* cuticular differentiation of the papilla ever occurred during the intermoult period, although a *spurious* appearance of segmentation, easily recognized as a fixation artefact, was observed in some preparations, fixed a day or two after the moult. No examination of the regenerates appearing at the second moult was made, but the observations on papillae suggested that they would be more or less symmetrical in size and in differentiation.

(iii) *Regenerate and Intermediate.*—A total of 524 animals, in 50 batches subjected to six different operating schedules, constituted the fraction of the experimental population in which every batch included one or more individuals producing "regenerate and papilla" at the first moult. This population included 22 animals, of which five came from one batch, two each from another four batches, and single examples from a further nine batches, in which an "intermediate" appeared on side II at the first moult. The regenerate on side I in these animals was normal, large, and complete, but that on side II ranged in appearance from an elongate, blastema-filled papilla with incipient segmentation visible on the cuticle (Plate 2, Fig. 11), quite different from the fixation artefacts mentioned above, to the almost complete regenerate with imperfect pretarsus seen in Plate 2, Figure 18. The absence of a properly formed pretarsus with claws formed a good criterion for distinguishing such "advanced" intermediates from the small and sometimes poorly differentiated, but essentially complete, regenerates occasionally found on side II in material of the "two regenerates" category (see below).

A surprisingly complete range of possibilities in the *proximodistal* differentiation of a blastema-filled papilla towards a complete regenerate was observed among the intermediates. Representative stages are shown in Plate 2, Figures 10-18. Most of these were represented by two or more examples, but the stage in Plate 2, Figure 16, was unique. Differentiation of the intermediates seemed to be very rapid, as shown especially by the history of the batch in which five intermediates were recorded from nine animals undergoing operations of schedule *D*. The first to moult did so on the eighth day of post-embryonic life, with a normal "regenerate and papilla" (Plate 2, Fig. 10). In the ensuing 24 hr the series of stages shown in Plate 2, Figures 11, 14, 15, 17, and 18, appeared. The sequence in which moulting occurred was readily deduced from the condition of the cuticle, ranging from very fresh, white, and soft in the animal of Plate 2, Figure 18, to completely hardened and darkened in the specimen of Plate 2, Figure 11. Intermediate stages in darkening corresponding to the sequence of differentiation of the intermediates were clearly recognizable in the animals of Plate 2, Figures 14, 15, and 17. The remaining animals moulted on the 10th day of post-embryonic life with normal pairs of "two regenerates" showing only mild asymmetry. Extremely rapid differentiation must have been in progress on side II during the ninth day of post-embryonic life, and the whole process of conversion of a fully organized (but not visibly differentiated) blastema into a complete regenerate cannot have occupied more than a few hours.

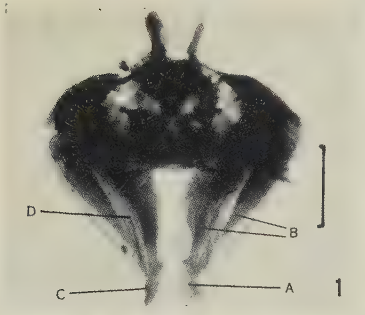
The rarity and theoretical interest of intermediates made it necessary to fix them as soon as seen, i.e. within a few hours of the first moult, and it has consequently been impossible to trace their differentiation and growth during the second instar, or to compare it with the distoproximal differentiation occurring in the side II papilla during the second instar of animals moulting with "regenerate and papilla."

Unpublished observations on teratological specimens indicated that many intermediates might be lost by autotomy early in the second instar—a further discouragement against attempting to follow their differentiation with the very limited number available. In an attempt to increase this number, and to test a hypothesis on the time relations of operations needed to secure the appearance of intermediates, a random assortment of animals aged between 0 and 2·5 days was subjected to successive operations separated by roughly 65 hr. The age at the first operation not being accurately known, this material has been excluded from the tables. It consisted of 74 individuals; 54 moulted with "regenerate and papilla," eight with "regenerate and intermediate," essentially similar in types to the stages shown in Plate 2, and 12 with "two regenerates." The proportion of intermediates, although increased, was not high enough for the attempt to be pursued further at this stage.

(iv) *Two Regenerates.*—This condition (Plate 1, Figs. 5 and 6) involved the appearance on each side, at the first moult, of a complete regenerate with tetramerous tarsus and fully formed pretarsus. The regenerate of side II was smaller in size and sometimes inferior in minor details of cuticular and muscular differentiation to that of side I. Combined asymmetry of size and differentiation sometimes gave rise to a rather "lop-sided" appearance (Plate 1, Fig. 5), but the presence of a fully formed pretarsus on each side readily distinguished all such examples from "regenerate and intermediate" material. At the other extreme (Plate 1, Fig. 6) were individuals in which the two regenerates were apparently identical in differentiation, and differed so little in size that measurement was necessary to establish that they were in fact asymmetrical.

Representative preparations of "two regenerates," derived from each of the operating schedules used, were examined in detail and a selection of them measured. They were also compared with preparations derived from animals undergoing unilateral or simultaneous regeneration. Evidently an adequate biometrical study of the symmetry of successive regenerates would require a more elaborate experimental design than the present work, and no firm conclusions were reached on the time relations of the varying degrees of symmetry observed. The size of the regenerate on side I, however, appeared to be rather constant for all operating schedules (except possibly *J*) and to exceed considerably the size of regenerates resulting from either unilateral or simultaneous operations. The regenerate of side II was much more variable in size, although always smaller than that of side I, but no consistent relationship was recognized between its size or differentiation and the time relations of the operations and the moult. In general, the sum of the lengths of the two successively produced regenerates was of the same order (5·5-6·5 mm) as that of two regenerates produced simultaneously. Occasional higher values were observed, up to 7 mm. Insufficient data are available for critical assessment of the importance of these exceptions.

REGENERATION AND MOULTING IN *BLATTELLA*. III



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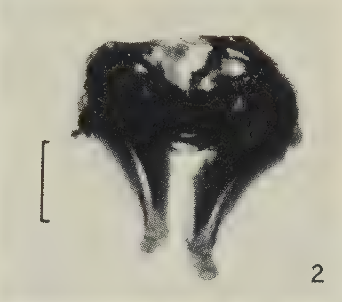

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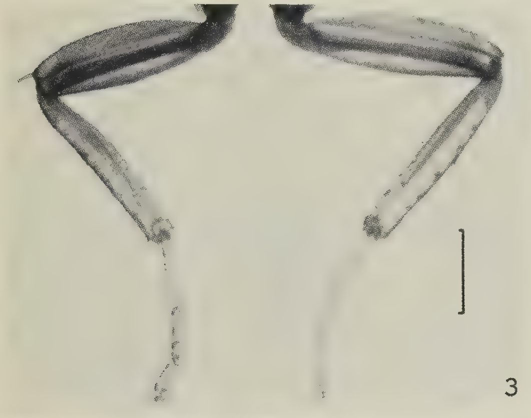

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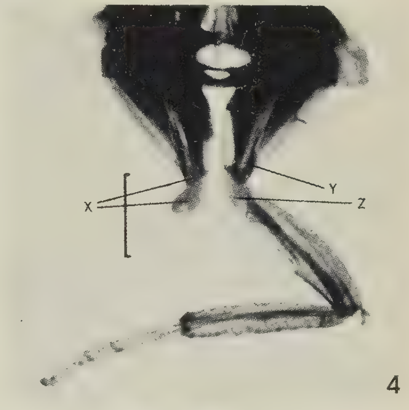

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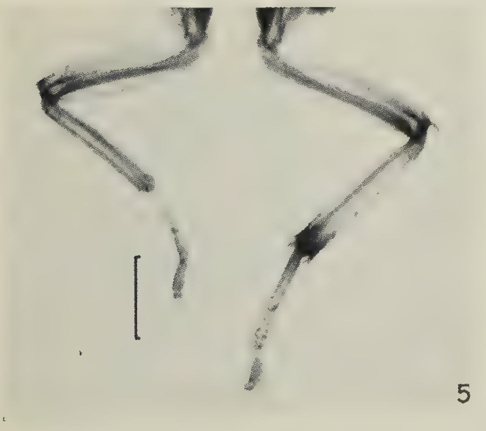

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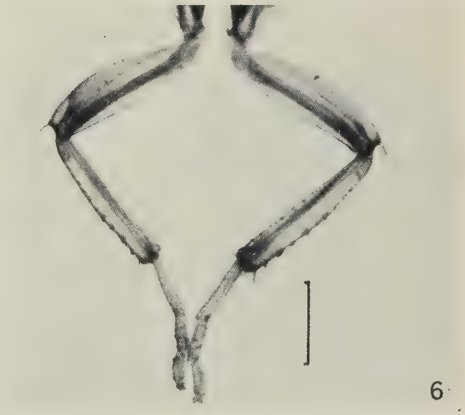

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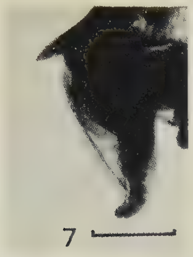
REGENERATION AND MOULTING IN *BLATTELLA*. III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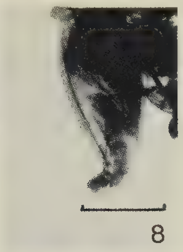
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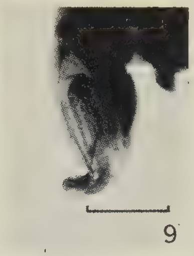
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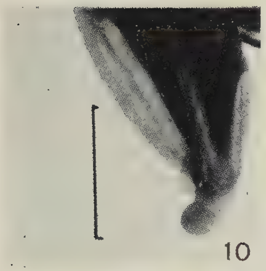
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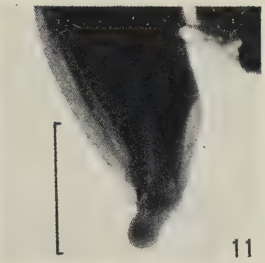
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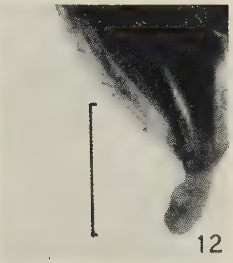
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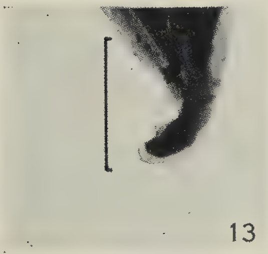
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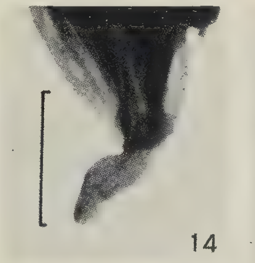
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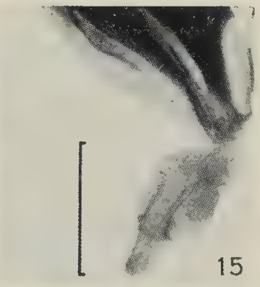
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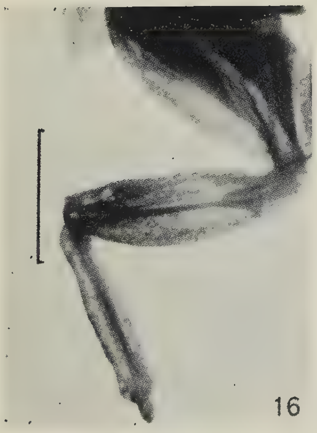
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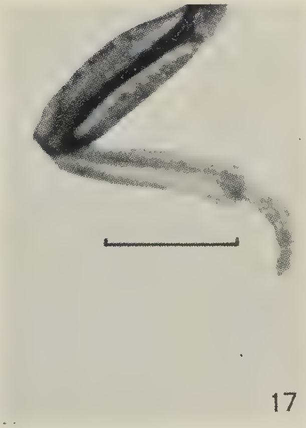
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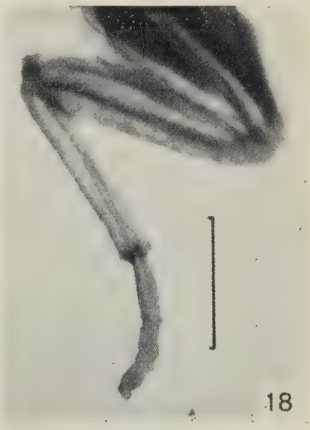
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The second moult of "two regenerates" material was observed only in a few individuals, from which no preparations were made. The symmetry of their regenerated metathoracic legs at the second moult appeared to be fairly complete, probably resembling that observed in the second moult of "two papillae" animals.

(*b*) *Influence of Successive Operations on the Time of Moulting*

The production of "two papillae" had no effect on the timing of the first moult, but the second moult was delayed significantly in comparison with that of the corresponding controls (Table 2). The magnitude of this delay was less than the age of the animals at the first operation, but was decidedly greater for schedule *J* than for *F* or *G*. The difference between the moulting of the material of schedules *F* and *G* was not significant, and the difference between either of them and schedule *J* was only doubtfully significant. Insufficient material of schedule *H* was available to permit their second moult to be investigated.

TABLE 2

EFFECT ON THE MOULTING CYCLE OF SUCCESSIVE OPERATIONS LEADING TO THE APPEARANCE OF TWO PAPILLAE AT THE FIRST AND TWO REGENERATES AT THE SECOND MOULT

Operating Schedule	Numbers of Animals Surviving at				Observed Delay in Moulting of Experimental Animals (days)*	
	First Moult		Second Moult			
	Experimentals	Controls	Experimentals	Controls	First Moult	Second Moult
F	19	85	17	60	No delay	0·96±0·18
G	70	144	59	100	No delay	1·11±0·13
H	11	40	N.r.†	N.r.†	No delay	N.r.†
J	125	137	92	78	No delay	1·58±0·13

* Computed by the method of Simpson and Roe (1939, pp. 192-3).

† Denotes "not recorded."

Where a "regenerate and papilla" appeared at the first moult, the moult was delayed (except in schedule *B*) by a period approximating the age of the animal at the *first* operation, irrespective of the timing of the second (Table 3). The schedule *B* animals behaved in a similar way to simultaneously regenerating animals undergoing operation on the first day of post-embryonic life (Stock and O'Farrell 1954), their moult being delayed to about the same extent as would have been expected had the first operation been performed on the second day of post-embryonic life.

The number of animals moulting with "regenerate and intermediate" was too small, and their distribution over the several schedules too scattered, for satisfactory tabulation of their moulting. In general, they tended to moult distinctly later than the animals producing "regenerate and papilla," so that the delay in their moulting was intermediate between the age at the first and at the second operation.

Production of "two regenerates" was associated, in all the schedules, with a delay in moulting approximating the age of the animal at the *second* operation, regardless of its age at the first (Table 4). Operation during the first day of post-embryonic life gave rise to no anomaly, the time of moulting being determined by the second operation in all "two regenerates" material of schedules *A* and *B*.

TABLE 3

DELAY IN THE FIRST MOULT ASSOCIATED WITH THE APPEARANCE OF A REGENERATE ON SIDE I AND A PAPILLA ON SIDE II, AFTER SUCCESSIVE OPERATIONS ON THE SCHEDULES SHOWN

Operating Schedules	Numbers of Animals Surviving at the First Moult		Observed Delay in First Moult of Experimental Animals (days)*	Age of Experimental Animals at the First Operation (days)
	Experimentals	Controls		
B	33	90	1·28±0·15	0-1
D and *E*	94	185	1·20±0·10	1-2
F, *G*, *H*	56	124	2·21±0·11	2-3

* Computed by the method of Simpson and Roe (1939, pp. 192-3).

TABLE 4

DELAY IN THE FIRST MOULT ASSOCIATED WITH THE APPEARANCE OF TWO REGENERATES, AFTER SUCCESSIVE OPERATIONS ON THE SCHEDULES SHOWN

Operating Schedules	Numbers of Animals Surviving at the First Moult		Observed Delay in First Moult of Experimental Animals (days)*	Age of Experimental Animals at the Second Operation (days)
	Experimentals	Controls		
A and *C*	109	129	2·37±0·10	2-3
B, *D*, *F*	279	383	3·39±0·06	3-4
E, *G*, *J*	134	235	4·20±0·10	4-5
H only	18	54	4·81±0·20	5-6

* Computed by the method of Simpson and Roe (1939, pp. 192-3).

IV. DISCUSSION

Omitting for the time being any consideration of the clearly exceptional animals moulting with "regenerate and intermediate," the bulk of the results reported can be discussed in the light of expectations derived from the "fresh start" hypothesis proposed by O'Farrell and Stock (1953), and on their assumption that regeneration interacts with the endocrine system controlling moulting, in such a way that there is a "critical period" in the moulting cycle, before which it can be subjected to a fresh start by regeneration and after which it cannot be so modified. On these assumptions, then, the following results are to be expected from successive operations:

Should the first operation occur *after* the critical period, neither it nor the second operation should influence the timing of the first moult, at which papillae should appear on both sides. The difference in the time available between operation and moulting for the healing of the cuticle etc. on the two sides should result in asymmetry of the two papillae, and that of side II should often show incomplete rounding off, with clotted blood indicating the presence of imperfectly healed cuticle on the trochanter at the moment of ecdysis. The "two papillae" results reported above accurately conform to these expectations. (A significant delay in the second moult of these animals cannot yet be clearly related to the general hypothesis under discussion, but might be expected in the light of observations on simultaneous regeneration (Stock and O'Farrell 1954). Such a delay, somewhat greater than in simultaneously regenerating animals, but not clearly related to the nature of the operating schedule, appears in Table 2. The resulting increase in the time available for growth and differentiation should enable side II to approximate towards symmetry with side I, as was in fact observed.)

If the first operation occurs *before* the critical period, it should set up a new moulting cycle dating from the day on which it is performed (except in animals undergoing operation during the first day of post-embryonic life, whose cycle will suffer an unexplained "excess" delay, as described in previous papers). Should the second operation then occur *after* the "secondary" critical period pertaining to this new cycle, it should not further influence the time of moulting. At the moult, a regenerate should appear on side I, and a papilla on side II, ecdysis being delayed by a period determined entirely by the age of the animal at the *first* operation. Table 3 shows the precise conformity with these expectations of the present results, even to the "excess" delay in moulting of schedule *B* material.

Where the second operation takes place *before* the onset of the "secondary" critical period set up by the first, a third or "tertiary" cycle should arise, associated with the appearance of two complete but more or less asymmetrical regenerates at the first moult. Assuming that the time relations of the "secondary" cycle are essentially similar to those of the original or "primary" cycle, the moult should then be delayed by a period approximating to the age of the animal at the *second* operation and uninfluenced by the timing of the first. Table 4 demonstrates the correctness of these expectations.

"Excess delay" in moulting, already observed in animals undergoing unilateral or simultaneous operations during the first day of post-embryonic life, appears in the present work only in "regenerate and papilla" material of schedule *B*, and not elsewhere. Apparently the "excess delay" does not persist through the "secondary cycle" to influence the "tertiary" one. This suggests a physiological equivalence, rather than a mere resemblance, between the effects on the moulting cycle of operations performed on the first and those done on the second day of post-embryonic life. Such an equivalence might result from the regeneration process failing to begin, or at least to take effect, until the second day.

Thus far, the results support the view that the "secondary cycle" not only exists, but represents a fresh start in time, a physiological "putting back the clock" such as can be explained most satisfactorily in terms of regeneration modifying the secretory rhythms of the neuro-endocrine complex controlling moulting.

After unilateral operation, the production of a regenerate requires a period approximating the normal duration of the instar (O'Farrell and Stock 1953). Successive operations leading to the appearance of "two regenerates" provide a longer time than this for development of the regenerate on side I, but those resulting in the production of "regenerate and papilla" clearly do not. In both categories, however, the rather constant size of the regenerate on side I is distinctly greater than that of regenerates resulting from either unilateral or simultaneous operations. Almost all the obvious variation observed is confined to side II in "two regenerates" material, where it is not recognizably related to the operating schedule nor to the time of moulting. These results suggest that neither of the successive regeneration processes exerts any inhibitory influence on the other. It further seems likely that the extra growth on side I may be related to physiological conditions associated with the early stages of the regeneration process on side II, since it is manifested in "regenerate and papilla" as well as in "two regenerates" material.

The foregoing discussion concerns the 98 per cent. of the experimental population conforming in the regeneration of both legs to the "all or nothing" principle of O'Farrell and Stock (1953). Exceptions to this principle occurred only on side II in the remainder of the experimental population. This provides the only justification for suggesting the existence of a real difference between the "primary" and the "secondary" moulting cycles. If the "secondary" critical period were somewhat less sharply defined than the "primary" one, a few individuals might undergo the second operation during a physiologically unstable phase. These animals would then moult with intermediates on side II, and at an age intermediate between the two expectations based on a strict application of the "all or nothing" principle. Table 1 suggests that this "secondary" critical period, although variable in its incidence, usually falls between 48 and 72 hr after the first operation. With a 65-hr interval between operations the proportion of intermediates obtained rose to 11 per cent., thus supporting this general interpretation. Even here, however, the occurrence of intermediates is rarer and less regular than would be expected if modification of the moulting cycle were the sole factor concerned. This leads to the suggestion that the extreme rapidity of conversion of the organized blastema into a visibly fully differentiated regenerate also reduces the probability of ecdysis occurring during the few hours in which the differentiating regenerate is in an intermediate condition. Such a hypothesis was put forward in partial explanation of the "all or nothing" principle by O'Farrell and Stock (1953), in preference to the idea that physiological (not necessarily visible) discontinuities arising in the hypodermis during a rapid differentiation process might inhibit moulting and hence prevent the appearance of intermediates. In the present work, a complete range of intermediates has appeared, such as could hardly arise if the process of differentiation were associated with hypodermal disturbances sufficient to prevent moulting. A fairly satisfactory explanation of the absence of intermediates on side I and their rarity on side II seems to be provided by the interdependence between the moulting cycle, with its short critical period, and the regeneration process, with its brief period of differentiation.

There is still no explanation, however, of the observation that differentiation of the regenerate on side II proceeds proximodistally when it occurs before the moult, but distoproximally in animals moulting with "regenerate and papilla," where differentiation on side II is postponed until early in the second instar. The existence of this reversal suggests the possibility that different phases of the moulting cycle may have different effects on the same stage of the regeneration process. Further elucidation of such relationships depends on knowledge of the sequence of events involved in the normal regeneration process and in the moulting cycle.

The present work seems to provide experimental evidence for the suggestion that the process of regeneration falls into three distinct phases. The first stage, occupying probably 2-4 days under the conditions of the present work, is that of dedifferentiation, mobilization of material, and organization of the blastema at the biochemical, but not at the visible, level. A few hours of very rapid visible differentiation constitute the second phase, at the end of which the essential cuticular structure and probably most of the muscular differentiation of the regenerate is complete. The last phase of 2-3 days duration seems to be occupied only by minor differentiation and by growth.

These three phases closely resemble the three phases in the moulting cycle postulated from experiments on unilateral and simultaneous regeneration, viz. pre-critical period (2-4 days), critical period proper (very short, occurring usually on the third or fourth day of post-embryonic life) and post-critical or pre-moult period, ending with the first moult 2-3 days after the critical period. Moreover, it has now been shown (Wickham 1954) that the prothoracic glands in *Blattella* undergo a sharp burst of mitotic activity, lasting at most a few hours, immediately before the appearance of mitoses in the hypodermis signals the physiologically irreversible onset of the moult. This burst of mitotic activity in the prothoracic glands approximately coincides in timing with the experimentally deduced primary critical period, and is preceded and followed by periods of 2-3 days of inactivity. This parallelism between the observed phases of regeneration as such, the phases of the moulting cycle deduced from experiments on regeneration, and the observed mitotic cycle of the prothoracic glands, demonstrates the complex possibilities available for interaction between regeneration and the neuro-endocrine system controlling moulting.

V. ACKNOWLEDGMENTS

We are indebted to the Research Committee of the New England University College (now the University of New England) for providing funds to assist this work. We should also like to thank Dr. H. G. Andrewartha for some stimulating discussion of the problems involved.

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EXPLANATION OF PLATES 1 AND 2

All preparations are photographed from the anteroventral aspect. Scales indicate 0·5 mm.

PLATE 1

Fig. 1.—"Two papillae" from operating schedule *J*, showing: (*A*) rounded, blastema-filled papilla of side I, with blastema extending into distal part of coxal musculature; (*B*) coxal musculature on side I showing distinct signs of disorganization; (*C*) trochanter on side II, angular, imperfectly healed with clotted blood and no clearly defined blastema; (*D*) coxal musculature on side II; disorganization has scarcely begun.

Fig. 2.—"Two papillae" from operating schedule *J*, showing complete healing on both sides. Asymmetry of dedifferentiation less marked than in Figure 1.

Fig. 3.—Pair of regenerates appearing at the second moult after operating schedule *J* had led to production of "two papillae" at the first. Note the nearly complete symmetry of the regenerates.

Fig. 4.—"Regenerate and papilla" from operating schedule *H*. Fixed at ecdysis, aged 8 days. Note (*X*) rounded, blastema-filled papilla on side II; coxal musculature modified, especially in distal region; (*Y*) unmodified coxal musculature of regenerate on side I; (*Z*) reestablished musculature of trochanter on side I.

Fig. 5.—"Two regenerates" from operating schedule *E*. Regenerates markedly asymmetrical.

Fig. 6.—"Two regenerates" from operating schedule *H*. Regenerates almost completely symmetrical.

PLATE 2

Figs. 7-9.—Sequence of differentiation on side II during second instar of "regenerate and papilla" material from operating schedule *E*. Moulted at 7-8 days old, fixed on second day after moult (Figs. 7 and 8), third day after moult (Fig. 9). Disto-proximal differentiation apparent.

Figs. 10-18.—Representative series of stages in proximodistal differentiation of intermediates appearing on side II at the first moult of "regenerate and intermediate" material. Figure 10 moulted at 7·5, Figure 13 at 9·5 days old, the rest during the ninth day of post-embryonic life. Figures 10, 11, 14, 15, 17, and 18 all come from one batch undergoing operation schedule *D*. Figure 12 is from schedule *D* in another batch, Figure 13 from *E*, and Figure 16 from *F*.

ANOMALIES IN RAT, MOUSE, AND RABBIT EGGS

By C. R. Austin*† and A. W. H. Braden*

[*Manuscript received June* 30, 1954]

Summary

Records were kept of 7284 rat eggs, 1120 mouse eggs, and 221 rabbit eggs. Amongst these were 22 rat eggs, 21 mouse eggs, and 1 rabbit egg that were unusual: "giant" eggs of about twice the normal volume, primary oocytes recovered from the fallopian tubes, probable instances of gynogenesis and parthenogenesis, eggs showing abnormalities of fertilization such as the presence of a single pronucleus or of a male and two female pronuclei, and 2-cell eggs with a binucleate blastomere. These anomalies are discussed and attempts are made to explain their occurrence.

I. Introduction

During the study of fertilization in rat, mouse, and rabbit eggs, a variety of spontaneously occurring anomalies were encountered. The commonest was polyspermy, and this has been made the subject of two previous communications (Austin and Braden 1953*a*, 1953*b*). The purpose of the present paper is to describe some of the rarer anomalies that have been seen. In all instances the eggs appeared viable and showed no sign of imminent degeneration.

II. Methods

Both rat and mouse eggs were recovered from the fallopian tubes, and then examined by phase-contrast microscopy, in the manner described by Austin and Smiles (1948). Ovulation was induced in immature rats by the method of Rowlands (1944), except that B.D.H. "Serogan" and "Gonan" were used as sources of gonadotrophins.

Rats were mated by two different procedures, referred to as "normal" mating and "delayed" mating. For normal mating, the females were caged with males overnight from the early afternoon and examined the following morning for copulation plugs. For delayed mating, oestrous females were placed with males for ½-1 hr at 10 p.m., 3 a.m., 7 a.m., or 11 a.m. and examined for copulation plugs. It has been shown that ovulation occurs in this colony between midnight and 4 a.m. (Austin and Braden 1954*b*).

III. Observations

(*a*) *Rat Eggs*

Eggs were obtained from the fallopian tubes of a total of 717 rats, of which 241 were normally mated adult rats, 370 were delay-mated adults, and 106 were

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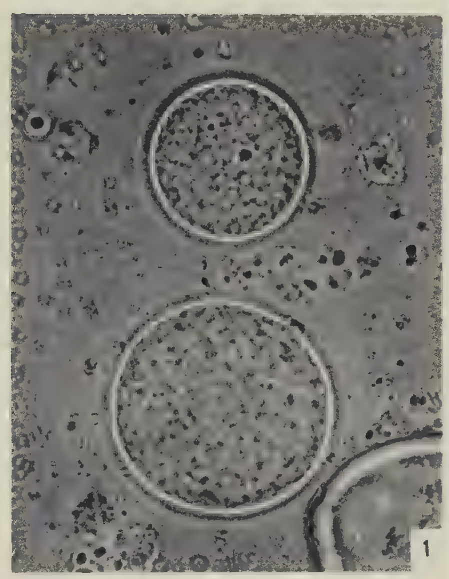
TABLE 1

DISTRIBUTION OF ANOMALOUS EGGS OBSERVED IN RATS, MICE, AND RABBITS

Mating		Number of Animals	Total Eggs	Anomalous Eggs						
				"Giant" Eggs	Primary Oocytes	Gynogenetic Eggs	Parthenogenetic Eggs	Abnormal Fertilization	Binucleate Blastomere	Total
Rats	Normal	241	2064	3	2	1	0	1	0	7
	Delayed	370	3481	5	3	1	2	1	1	13
	Normal (immature rats)	106	1739	1	1	0	0	0	0	2
	Totals	717	7284	9	6	2	2	2	1	22
Mice	Normal and delayed	147	1120	1*	15	0	0	4	1	21
Rabbits	Normal and delayed	27	221	1	0	0	0	0	0	1

*This egg was also a primary oocyte.

ANOMALIES IN RAT, MOUSE, AND RABBIT EGGS



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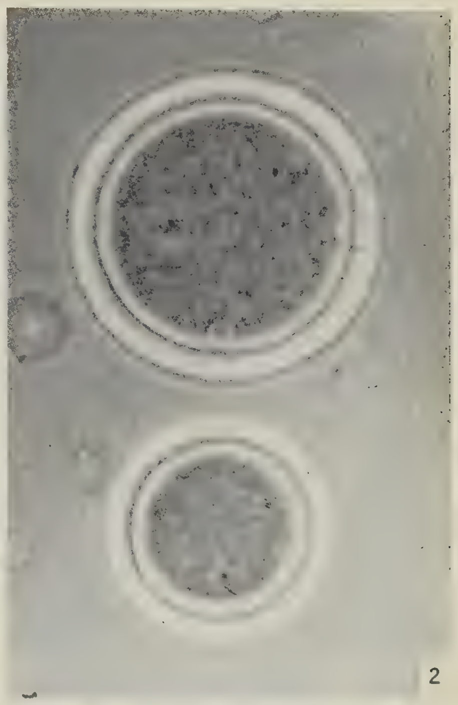

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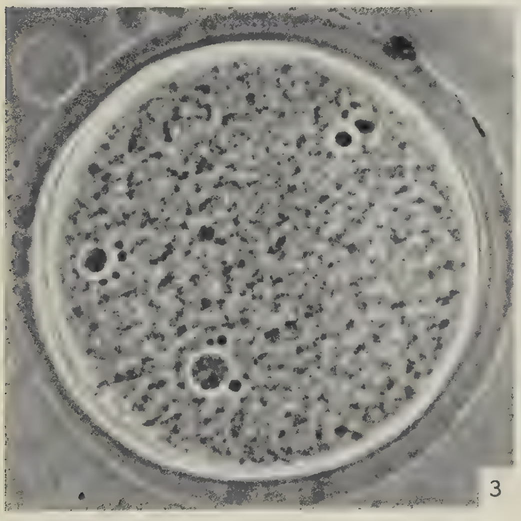

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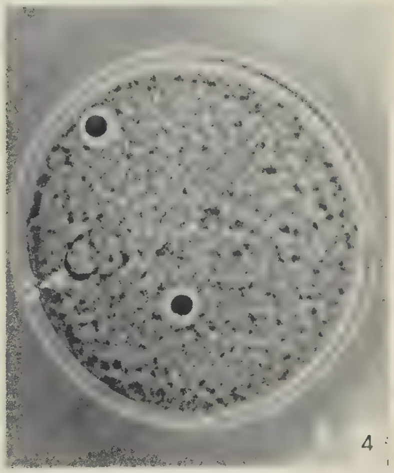

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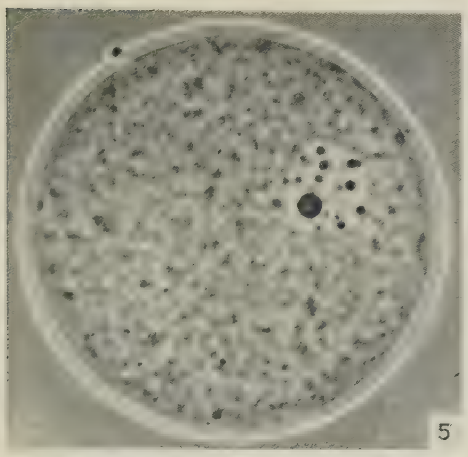
ANOMALIES IN RAT, MOUSE, AND RABBIT EGGS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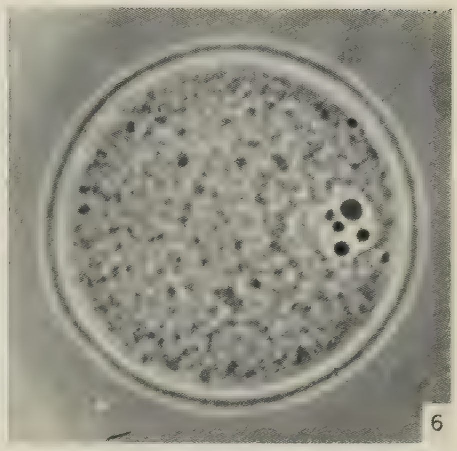
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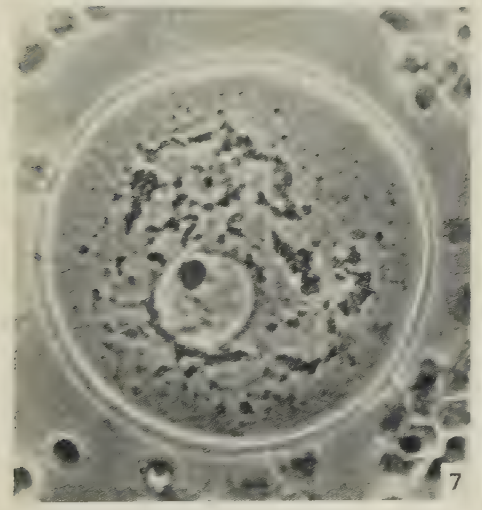
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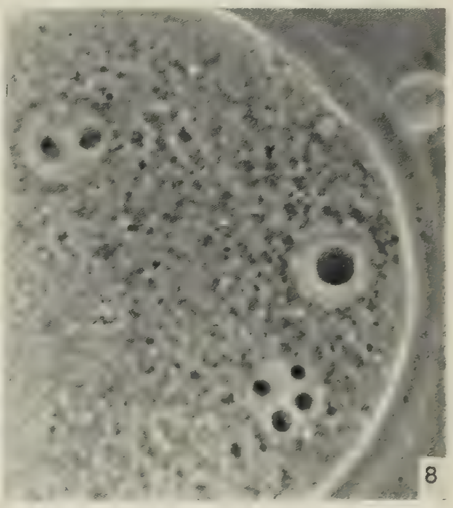
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normally mated immature rats. The total number of eggs recovered was 7284 and amongst these there were 22 eggs that presented unusual features (Table 1).

(i) *Giant Eggs.*—Nine "giant" eggs were seen; they were quite distinctive, for their diameter was about 1·3 times that of normal eggs so that their volume was about twice that of normal eggs (Plate 1, Fig. 1). Seven of the "giant" eggs contained sperms and were undergoing fertilization. Two of these were polyspermic (dispermic), and one was monospermic but had two female pronuclei (Plate 1, Fig. 3); in the remaining four eggs the process of fertilization appeared to be quite normal. A single metaphase chromosome group was observed in the two unpenetrated "giant" eggs, but the chromosome number could not be determined.

(ii) *Primary Oocytes.*—The primary oocyte was easily recognized by its large spherical nucleus, usually with only a single large nucleolus, and by the characteristic texture of the cytoplasm. Six oocytes were recorded in the present series, two from the normally mated rats, three from the delay-mated, and one from the immature rats. One oocyte had been penetrated by six sperms which all lay in the perivitelline space, none having entered the vitellus.

(iii) *Early Gynogenesis.*—Two eggs appeared to be undergoing gynogenetic development, or more specifically gyno-merogeny. In each of them, the sperm mid-piece lay, loosely coiled, in a small mass of cytoplasm that was clearly separate from the rest of the vitellus. The sperm head had changed and given rise to a small ill-formed pronucleus, and the acrosome could be seen close by. One of the eggs was undivided; it had a polar body and a single large nucleus, resembling in size and form a male pronucleus. The other egg was in the 2-cell stage; the cytoplasm appeared normal but the nuclei were smaller than usual and had only one nucleolus each (Plate 1, Fig. 4).

(iv) *Rudimentary Parthenogenesis.*—Two eggs were recovered, each of which had a large, well-formed nucleus, but showed no evidence of sperm penetration (Plate 2, Figs. 5 and 6). The nuclei, particularly the one shown in Plate 2, Figure 5, bore a strong resemblance to male pronuclei both in size and structure. Most of the other eggs that were obtained from the same fallopian tubes as these two had been penetrated by sperms and were in the course of apparently normal fertilization.

(v) *Abnormal Fertilization.*—Two eggs were seen that had been penetrated by sperms and were undergoing apparently normal fertilization except that they had two distinct female pronuclei in addition to a male pronucleus. One of these was a "giant" egg and has already been described (Plate 1, Fig. 3). The other was of normal size; it had no polar body. A photograph of this egg was published in an earlier communication (Austin and Braden 1953*b*, Plate 3, Fig. 18). A third penetrated egg had a single nucleus resembling a normal male pronucleus; no egg chromosomes could be distinguished.

(vi) *Two-cell Egg with Binucleate Blastomere.*—One 2-cell egg was seen that contained a single sperm and appeared quite normal, except that there were two well-formed nuclei in one blastomere. A picture of this egg was also published in the paper just referred to (Plate 3, Fig. 20).

(*b*) *Mouse Eggs*

From 147 mated mice, a total of 1120 tubal eggs was obtained, of which 21 were anomalous (see Table 1). The majority (16) of these were primary oocytes (Plate 2, Fig. 7), including one that was of "giant" proportions, and resembled in nuclear structure the primary oocyte of the rat; the egg shown had been penetrated by four sperms, which were all in the perivitelline space. The corona radiata in these eggs was much less dense than that of the normal eggs in the same tube. The other anomalies were: three penetrated eggs that contained only one pronucleus each, another egg with two well-formed female pronuclei in addition to a male pronucleus, and a 2-cell egg that had two nuclei in one of the blastomeres but was otherwise normal (Plate 2, Fig. 8).

(*c*) *Rabbit Eggs*

Among 221 eggs obtained from 27 mated rabbits there was one "giant" egg, which, like those observed among the rat eggs, had about twice the normal volume (Plate 1, Fig. 2).

IV. Discussion

The origin of the "giant" eggs is enigmatic. They may have resulted from overgrowth of a normal oocyte while in the follicle, but the fact that they were regularly about twice the volume of normal eggs suggests an origin early in oogenesis. They may have been initially tetraploid, but this possibility was not investigated. "Giant" eggs appear to be more prone to irregularities of fertilization, for, out of the seven fertilized rat eggs seen, two were polyspermic and another had two female pronuclei. If the "giant" eggs were indeed diploid after meiosis, these irregular forms may have been capable of giving rise to tetraploid and pentaploid embryos.

The presence of primary oocytes in the fallopian tube may result from the accidental ovulation of a normal, but immature, follicle or from the ovulation of a follicle in which maturation of the egg alone has been delayed. There appears to be no earlier report of the occurrence of tubal primary oocytes in rats or mice. Longley (1911), however, described an oocyte that was recovered from the fallopian tube of a cat, and the ovulation of primary oocytes is evidently normal for the dog (Van der Stricht 1923), the fox (Pearson and Enders 1943), and the horse (Hamilton and Day 1945). In the present series, oocytes were seen much more frequently in mice than in rats, but the reason for this is obscure. The fact that one rat oocyte had been penetrated by six sperms and one mouse oocyte by four sperms is of particular interest because there is evidence that the membranes of normally ovulated rat eggs must undergo a form of maturation in the fallopian tube before sperm penetration can occur (Austin and Braden 1954*b*). Evidently the same kind of change can take place in the outer membranes of an oocyte after ovulation even though the vitellus is in an immature state. The presence of all the penetrating sperms in the perivitelline space suggests that the immaturity of the vitellus precluded the entry of sperms into it. Lack of maturity in the vitellus is apparently not a bar to sperm entry in all species, however, for in the dog, penetration occurs before the first meiosis (Van der Stricht 1923).

There seems little doubt that two of the eggs seen were indeed undergoing a form of gynogenesis, for the sperm mid-piece and acrosome, as well as the ill-formed male pronucleus, could be seen in a clearly separated portion of the vitellus. It appears likely that the sperm, having entered the vitellus and thus provided the activating stimulus, became accidentally included in an extrusion of the cytoplasm. As one of these eggs had undergone cleavage, it seems that, in the rat, development may proceed at least to the 2-cell stage without the normal involvement of the male pronucleus. The relatively small size of the nuclei in this egg is more likely due to their recent formation than to a haploid condition, although it has been shown that eggs that are probably haploid have nuclei of slightly less than the normal size (Austin and Braden, unpublished data). The occurrence of gynogenesis in mammals has previously been described only after irradiation of the sperm (Amoroso and Parkes 1947).

Rudimentary parthenogenesis seems an appropriate designation for the process displayed by two other eggs in this series. Neither contained a sperm, yet each had a large, well-formed nucleus. The formation of a single nucleus after artificial stimulation has been reported to occur in some rabbit eggs (Thibault 1949), and recently it has been found possible to produce this result in rat eggs (Austin and Braden, unpublished data). It is interesting that the nuclei, particularly in one egg (Plate 2, Fig. 5), resembled male pronuclei in size and structure. This suggests that there is, in normal fertilization, some competition between the pronuclei for nuclear constituents which plays a part in limiting the size of the female pronucleus. The same explanation may account for the size and form of the nucleus in the undivided gynogenetic egg.

With both rats and mice, the eggs that exhibited abnormal fertilization had either a single pronucleus, or two female pronuclei in addition to the male pronucleus. In the eggs with a single pronucleus, it seems likely that this was the male and that scatter of the chromosomes was the reason for the non-development of the female pronucleus. Such a result has been observed in eggs subjected to artificial stimulation (Austin and Braden 1954*a*), and spontaneous chromosome scatter may be associated with long-delayed sperm penetration. On the other hand, Pesonen (1949) considered that, in mouse eggs, the male and female pronuclei may occasionally unite to form a single nucleus. When two female pronuclei are present in addition to the male pronucleus, the mechanism probably involves inhibition of second polar body formation, such as has been induced by appropriate treatment before sperm penetration in both *Triturus* eggs (Fankhauser and Godwin 1948) and rat eggs (Austin and Braden 1954*a*). Presumably such eggs could give rise to triploid embryos as suggested by Fischberg and Beatty (1952) for mice.

It is difficult to account for the binucleate blastomeres seen in 2-cell eggs. Van der Stricht (1910) noted a similar condition in the egg of a bat. In frog eggs, binucleate blastomeres are seen when polyspermic fertilization has occurred (Wilson 1925) but this explanation does not appear valid for the rat egg (Austin and Braden 1953*b*).

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EXPLANATION OF PLATES 1 AND 2

Photographs were taken with the aid of a phase-contrast microscope.

PLATE 1

Fig. 1.—A "giant" rat egg compared with an egg of normal size. Both are undergoing fertilization. × 170.

Fig. 2.—A "giant" rabbit egg compared with an egg of normal size. Neither has been penetrated by sperms. × 170.

Fig. 3.—A "giant" rat egg showing abnormal fertilization: there are two female pronuclei in addition to the male pronucleus. × 350.

Fig. 4.—A 2-cell rat egg showing supposed early gyno-merogeny. The small size of the nuclei is evident and part of the coiled sperm mid-piece(*s*) may also be discerned. Compression of the egg has obscured the individuality of the blastomeres and of the cytoplasmic extrusion containing the sperm mid-piece and male pronucleus. × 350.

PLATE 2

Figs. 5 and 6.—Two rat eggs exhibiting rudimentary parthenogenesis. In each there is a single well-formed nucleus; neither egg contains a sperm. × 350.

Fig. 7.—The primary oocyte recovered from the fallopian tube of a mouse. This egg has four sperms in the perivitelline space but none within the vitellus. The scarcity of coronal cells is in contrast to the density of these cells about oocytes recovered from mature follicles. × 350.

Fig. 8.—A 2-cell mouse egg with a single nucleus in one blastomere and two apparently normal nuclei in the other. × 650.

THE NUMBER OF SPERMS ABOUT THE EGGS IN MAMMALS AND ITS SIGNIFICANCE FOR NORMAL FERTILIZATION

By A. W. H. Braden* and C. R. Austin*†

[*Manuscript received April* 15, 1954]

Summary

Figures are given for the numbers of sperms at the site of fertilization in normally mated rats, mice, and sheep, and also in rats and rabbits mated at the time of ovulation or later. For the sheep, data are also given on the distribution of sperms throughout the female genital tract.

In the four species investigated, the degree of restriction exercised by the female genital tract on sperm passage varied between species in such a way that the number of sperms at the site of fertilization tended to be inversely related to the size of the site. The concentration of sperms at the site would therefore presumably vary relatively little between species.

It is shown that, in normally mated rats, higher concentrations of sperms at the site of fertilization were significantly associated with the presence of larger numbers of sperms within the eggs.

Experimental evidence thus supports the concept that the usual concentration of sperms at the site is high enough to give all the eggs a good chance of being penetrated before the end of their fertile life, but not so high that there is much risk of a disturbance of fertilization through the entry of excess sperms.

The frequency of successful sperm-egg collisions was estimated to be one every 2 min for the rabbit and one every 10 min for the rat.

The restrictive action of the female genital tract in the rat is evidently relaxed towards the end of oestrus, for sperm passage is then facilitated.

I. Introduction

Special significance attaches to the number of sperms that gather after coitus at the site of fertilization, for the magnitude of this number will influence not only the chances for the meeting of egg and sperm, but also the chances for the entry of an excess of sperms into the eggs. The chances of an egg being penetrated by an excessive number of sperms will, of course, also depend upon the changes undergone by the zona pellucida and the surface of the vitellus in consequence of sperm penetration; these are discussed elsewhere (Austin and Braden 1953*a*, 1953*b*; Braden, Austin, and David 1954).

Data on the number of sperms at the site of fertilization in the rat, rabbit, and ferret were reviewed in a recent communication (Austin and Braden 1952); the numbers reported were all relatively small and it was suggested that an important function of the female genital tract was to restrict the number of sperms that accumulate in the vicinity of the eggs. More detailed figures for the rabbit were supplied by Braden (1953) and these further supported the idea that the female tract has a restrictive function.

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Information is now available on two other species of mammals, the mouse and the sheep, and a better understanding has also been gained of the mechanism of the restrictive function in the rat, rabbit, and sheep, and of the manner in which it varies in relation to the time of coitus and ovulation.

II. METHODS

Adult albino and "hooded" rats were allowed to copulate at certain periods before, during, or after ovulation. The animals were slaughtered 1-20 hr after coitus and the number of sperms in the distended portion of the ampulla was counted by the method previously described (Austin 1952). Female albino mice were run continuously with males and those with copulation plugs in the morning were killed during the course of the day. The number of sperms in the distended part of the ampulla was determined by a similar method to that used for the rats.

Ovulation was induced in cross-bred rabbits by the intravenous injection of 40 I.U. of "Gonan,"* and, 10 hr later, the animals were each allowed to copulate with two males. Sperm counts were made by the method employed by Braden (1953).

Merino ewes were run continuously with a raddled ram and inspected for marking each morning and evening. Animals that were marked were killed 24-48 hr after the estimated time of coitus. The numbers of sperms in the various parts of the genital tract were estimated by a technique similar to that used for the rabbit.

TABLE 1

NUMBER OF SPERMS AT THE SITE OF FERTILIZATION IN RATS KILLED AFTER NORMAL MATING

Time of Killing (Hr. after Ovulation)	Number of Rats	Number of Sperms per Tube	
		Mean	Range
0	46	24	0-134
8	30	44	0-164
12	30	45	4-160

III. OBSERVATIONS

(a) *Number of Sperms at the Site of Fertilization*

(i) *Rats*.—The number of sperms in the distended part of the fallopian tube was determined in 105 rats that had been permitted to mate freely under normal colony conditions. Generally, copulation occurred in the late afternoon and was seldom observed later than 8 p.m.; ovulation took place between midnight and 4 a.m. (Austin and Braden 1954). In 48 rats with copulation plugs, killed between 1 a.m. and 4 a.m. on the day of ovulation (day 1), a mean number of 24 sperms was found in individual fallopian tubes (Table 1). Another group of rats was killed between 11 a.m. and noon of day 1, and in these animals the mean number of sperms in each fallopian tube was 44. There is a significant difference ($P < 0\cdot01$) between these two means. A third group of rats was killed between 3 and 4 p.m. on day 1, and the mean number of sperms in these was 45 (excluding one abnormal count of 332).

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* A preparation of chorionic gonadotrophin supplied by British Drug Houses Ltd.

TABLE 2

NUMBER OF SPERMS AT THE SITE OF FERTILIZATION IN RATS MATED DURING AND AFTER OVULATION

Time of Mating			Hours after Mating							
			1	2	2½	3	4	5	6	7
3 a.m.	Number of rats		—	15	—	10	—	10	—	10
	Number of sperms per tube	Mean:	—	27	—	57	—	47	—	63
		Range:	—	0-131	—	0-197	—	8-149	—	9-188
7 a.m.	Number of rats		10	15	10	10	10	10	10	—
	Number of sperms per tube	Mean*:	7	21	33	32	50	55	60	—
		Range:	0-23	0-81	4-107	4-131	0-152	0-267	15-167	—
11 a.m.	Number of rats		—	10	10	10	—	10	—	10
	Number of sperms per tube	Mean:	—	53	92	115	—	94	—	79
		Range:	—	5-152	3-317	6-345	—	0-312	—	5-188

* The mean figures for the 7 a.m. mating have already been reported (Austin 1952).

Determinations were also made of the sperm numbers at the site of fertilization in rats that were not permitted to mate until the time of ovulation or later. Oestrous female rats were selected by examination of the vaginal smear and placed with males for half an hour from 3 a.m., 7 a.m., or 11 a.m. Those that mated were recognized by the presence of copulation plugs. Coitus generally occurred in the first 15 min of the period; for convenience, therefore, the mating times are referred to as 3 a.m., 7 a.m., and 11 a.m. respectively. Altogether, 170 mated rats were obtained in this way. The results are set out in Table 2. In the rats mated at 3 a.m. the mean number of sperms per fallopian tube was found to increase from 27 at 2 hr to 57 at 3 hr; thereafter there was no significant change. The mean number of sperms in the tubes of rats mated at 7 a.m. increased from 7 at 1 hr to 50 at 4 hr. The mean values for 5 and 6 hr were similar to those obtained at 4 hr. In the rats mated at 11 a.m. the mean number of sperms per tube was 53 at 2 hr and 92 at 2½ hr after mating; after 2½ hr the mean values did not change significantly. Thus the mean number of sperms per tube increased for 2½-4 hr after delayed mating, and higher figures were observed after mating at 11 a.m. than at 3 a.m. or 7 a.m.

(ii) *Mice.*—As in the rat, fertilization takes place in a distension of the ampulla (Sobotta 1895). Counts of the number of sperms at this location about 10-15 hr after ovulation were made in 15 mice. The mean number was 16·9 sperms per tube (range 2-75).

TABLE 3

NUMBER OF SPERMS IN THE FALLOPIAN TUBES OF RABBITS KILLED AFTER COITUS PERFORMED AT THE TIME OF OVULATION

Hr after Coitus	Number of Rabbits	Number of Sperms in Tubes		
		Entire Tubes	Ovarian 2/3	Ovarian 1/3
4	7	1775 (520-2592)*	132 (0-444)	20 (0-64)
5	4	2335 (1450-3296)	451 (138-1136)	77 (0-200)
6	6	5387 (1616-11,282)	1166 (68-4,010)	228 (0-550)

* Mean value (per rabbit) and range of values.

(iii) *Rabbits.*—Estimates of the number of sperms in the fallopian tubes of untreated rabbits at intervals after mating have already been reported (Braden 1953). Similar counts have now been made for rabbits allowed to copulate 10 hr after the injection of chorionic gonadotrophin, i.e. at about the time of induced ovulation (Table 3). The mean number of sperms per rabbit in the entire tubes was found to increase from 1775 at 4 hr to 5387 at 6 hr after mating. These values are very similar to those found in untreated animals killed 4 and 6 hr after mating (Braden 1953). In the ovarian two-thirds of the tubes, which includes the site of fertilization, the mean number of sperms increased from 132 at 4 hr to 1166 at 6 hr after coitus.

(iv) *Sheep.*—The distribution of sperms in the genital tracts of six ewes was examined 24-48 hr after coitus. The mean number of sperms per animal in the entire fallopian tubes was 15,075 (range 1,500-23,700 sperms) and in the ovarian two-thirds of the tubes, the approximate extent of the ampulla, it was 673 (range 72-2700 sperms). For the ovarian third alone, the mean number present was 184 (range 40-524 sperms). In the caudal and cranial halves of the uterine horns, mean numbers of 254,000 (range 40,000-1,170,000) and 171,000 (range 5,000-560,000) sperms, respectively, were found. The mean number recovered from the cranial half of the vagina was $1 \cdot 81 \times 10^6$ sperms (range $0 \cdot 2 \times 10^6$ to $6 \cdot 0 \times 10^6$).

(*b*) *Number of Sperms that Penetrate the Eggs in Rats*

Sixty-eight female rats mated under normal colony conditions, and killed between 11 a.m. and 3 p.m. on the day of ovulation, yielded 627 eggs. There were 614 penetrated eggs, of which 133 (21·7 per cent.) contained more than one sperm. Examination of the data revealed that relatively large numbers of sperms at the site of fertilization were often associated with increased numbers of sperms within the eggs. The information is best considered in terms of individual fallopian tubes rather than entire rats, because the two tubes in any one rat represent two different sets of circumstances and often vary in the results they yield. From the 68 rats killed, five tubes were recovered that contained one or more unpenetrated eggs; the eggs in the remaining 131 tubes were all penetrated. Among the 131 tubes, there were 50 in which the eggs contained only one sperm each and in these tubes the mean number of sperms about the eggs was 35·7. In each of another 35 tubes, one of the eggs had two sperms within the zona pellucida; there was thus one sperm in excess of requirements in the eggs in each tube. The mean number of sperms about the eggs was 39·4. The data were sorted in the same way for tubes in which there were 2, 3, 4-5, 6-8, and more than 8 extra penetrating sperms. The results are in Table 4. Most of the sperms that have been referred to as being in excess of requirements were seen in the perivitelline space of the eggs; 10 eggs, however, had one extra sperm in the vitellus and were therefore polyspermic (dispermic).

TABLE 4

NUMBER OF EXTRA SPERMS IN THE EGGS OF ANY ONE FALLOPIAN TUBE AND ITS RELATION TO THE NUMBER OF SPERMS ABOUT THE EGGS, AND THE NUMBER OF POLYSPERMIC (DISPERMIC) EGGS

Number of Extra Sperms per Tube	Number of Tubes	Number of Sperms About the Eggs		Total Number Penetrated Eggs	Dispermic Eggs	
		Total	Mean per Tube		Total	Per cent.
0	50	1785	35·7	211	0	—
1	35	1379	39·4	174	3	1·7
2	17	749	44·1	80	2	1·5
3	10	563	56·3	42	1	2·4
4-5	11	720	65·5	59 } 107	3	2·8
6-8	6	399	66·5	30		
>8	3	408	136	18		

A regression analysis (Table 5) was carried out of $y = \log y^1$ against x, where $y^1 =$ the number of sperms at the site of fertilization and $x =$ the total number of extra sperms in the eggs of any one tube. A highly significant association ($P < 0{\cdot}01$) was found between the two values.

There appears to be a positive association between the incidence of polyspermy and the presence of higher numbers of extra sperms within the eggs of any one tube. The data (Table 4), however, are insufficient for any definite conclusion to be reached.

IV. Discussion

It is generally conceded that the site of fertilization in mammals lies in the ampulla of the fallopian tube. The size of the site varies, however, between species; in some it is restricted to a small distension of the ampulla, e.g. the rat (Tafani 1889); the mouse (Sobotta 1895); the guinea pig (Lams 1913); and the elephant shrew (Van der Horst and Gillman 1941); while in others it may involve the whole ampulla, as in the rabbit (Braden 1953). Only for a few mammals has it been suggested that fertilization takes place elsewhere than in the ampulla. In the Tenrecidae — *Hemicentetes* (Bluntschli 1938), *Ericulus* (Strauss 1938), and *Centetes* (Strauss 1950) — fertilization is reported to occur in the follicle. Pearson (1944) considered the same to be true for the short-tailed shrew; Brambell (1935), however, stated that in the common shrew the ampulla is the site of fertilization. In the ferret, Hammond and Walton (1934) thought that the eggs were fertilized in the ovarian capsule, but this seems unlikely, for Enders (1952), who worked with another member of Mustelidae, the mink, observed that the eggs were penetrated by sperms only after they entered the ampulla.

Table 5

REGRESSION ANALYSIS OF THE NUMBER OF SPERMS AT THE SITE OF FERTILIZATION AGAINST THE NUMBER OF EXTRA SPERMS IN THE EGGS

Source of Variation	Mean Square	Degrees of Freedom	F
(1) Slope	2625	1	15·19*
(2) Array of means about regression	0·082	6	—
(3) Residual	0·173	120	

* $P<0\cdot01$.

The numbers of sperms that were found at the site of fertilization in four species of mammals differed widely. In rats killed at the time of ovulation the mean number was 24 sperms per tube, and in those killed 8-12 hr after ovulation the mean number was observed to be 45 sperms. These results are consistent with an earlier report of 43 sperms per tube in rats killed after ovulation (Austin 1948), and differ little from the findings of Blandau and Odor (1949), who noted means of 12 and 30 sperms in rats killed 12 and 24 hr, respectively, after mating. The mean number of sperms in the mice used in the present investigation was 17 per tube. In strong contrast to the figures for rats and mice are those for rabbits (Braden 1953) and sheep, namely, about 500 and 340 sperms per tube, respectively.

The number of sperms at the site of fertilization in all these animals is very small compared with the number introduced into the female tract at coitus. As has already been pointed out (Austin and Braden 1952), this reflects a restrictive action of the female genital tract on the passage of sperms to the site of fertilization. The data obtained on the distribution of sperms in the genital tract of the ewe further illustrate the relative importance of various parts of the tract in this restrictive mechanism. In the sheep, the influence of the cervix far surpasses that of other structures: the normal ram ejaculate contains about 3000×10^6 sperms (Terrill 1937), but less than a million of these pass the cervix. In the rabbit (Braden 1953), on the other hand, the utero-tubal junction is the most efficient part. The cervix of the ewe is structurally well adapted for this function: it is about 4 cm long and the lumen is practically closed by mucosal folds (Sisson and Grossman 1940). The numbers of sperms found in the uterus and tubes of ewes in the present study are somewhat higher than figures published by Warbritton *et al.* (1937). They found a mean number of 26,350 (range 130-91,300 sperms) in the uteri of seven ewes killed 11 hr after mating, and a mean number of 6860 (range 8-22,500 sperms) for the entire tubes. The difference between these and the present results (425,000 and 15,075 sperms, respectively) is probably due to variations in the technique used for estimating sperm number.

The magnitude of the number of sperms at the site of fertilization is of importance, for it influences the chances of meeting of egg and sperm, and thus also the chances of sperm penetration into the egg. Other variables also affect the chances of meeting of sperm and egg but these are not easy to measure. Nevertheless, an approximate assessment of the frequency of sperm-egg collisions in the rabbit and rat may be made in the following way. The zona pellucida of the rabbit does not become less penetrable after the entry of the first sperm (Braden, Austin, and David 1954), so that the rate at which sperms accumulate within the bounds of the zona will give an indication of the collision frequency. Rabbits that were killed 13 hr after normal mating yielded eggs containing a mean number of 18 sperms each (Braden, Austin, and David 1954). As ovulation begins about 9½ hr after mating and the mean number of eggs recovered at 13 hr was 7·5, it follows that effective sperm-egg collisions must have occurred at an average rate for the 3½ hr of about one every 2 min. The rate was probably somewhat slower than this when penetration was just beginning, for the data indicate a progressive increase in the rate of effective collisions up to 6 hr after ovulation.

The same method of calculation cannot be used for the rat, for in this species the zona loses its penetrability to sperms within 1½-2 hr after the entry of the first sperm (Braden, Austin, and David 1954). However, it has been shown that, in rats mated at 7 a.m., the penetration of all the eggs in any one tube took about 1 hr (Austin and Braden 1954). When 40-60 per cent. of the eggs in any one tube had been penetrated, the penetrated eggs contained an average of 1·3 sperms (unpublished data). As the mean number of eggs was 4·8, it follows that about three successful sperm-egg collisions must have occurred during the first ½ hr after penetration had begun, so that the collision rate may be stated as about one every 10 min. Thus, sperm-egg collisions occurred five times more frequently in rabbits than in rats. Since the rabbit egg has roughly four times the surface area of the rat egg, the collision frequency per unit of surface area would be about the same. Other things being equal, this would mean that the sperm concentration was of the same order in the two species. Evidently the effect of the greater sperm number in the rabbit compensated for the larger size of the site of fertilization. Consistent is the fact that in the mouse a small number of sperms is associated with a small site, and in the sheep a large number with a large site.

These considerations suggest that the restrictive action of the female genital tract on sperm passage varies between species in such a way as to bring the frequency of sperm-egg collisions within a certain range, in spite of the size differences of the site of fertilization. This range is such that all eggs will have good chances of being fertilized, while at the same time there is little danger of the entry of an excess of sperms into the eggs. The data on the rat show that, even under normal circumstances, large numbers of sperms at the site of fertilization are associated with an increased frequency of sperm-egg collisions, resulting in larger numbers of sperms in the perivitelline space, and possibly also in an increased incidence of polyspermy. Both the presence of numerous sperms in the perivitelline space and polyspermy could be pathological. In the rat and the pocket gopher, eggs that had an unusually large number of sperms in the perivitelline space were clearly degenerate (Braden, Austin, and David 1954; Mossman and Hisaw 1940). Polyspermy probably leads to polyploidy in the embryo (Austin and Braden 1953*b*) and polyploid embryos, as far as is known, seldom go to term (Beatty 1951).

As already mentioned, the number of sperms at the site of fertilization in normally mated rats was found to be significantly higher 8 and 12 hr after ovulation (44 and 45 sperms per tube) than at the time of ovulation (24 sperms per tube). Furthermore, in rats mated during the period of ovulation (at 3 a.m.) and a few hours after ovulation (at 7 a.m.) the mean sperm number reached a level of 50-60 at 3-4 hr after mating. Even higher mean values, up to 115 sperms per tube, were seen when the rats were mated at 11 a.m. These observations indicate that the restriction on the passage of sperms through the female genital tract of the rat is relaxed towards the end of oestrus. On the other hand, in the rabbit no such relaxation was detected, for the numbers of sperms in the fallopian tubes of animals mated at the time of ovulation were very similar to those already reported (Braden 1953) for normally mated rabbits.

V. ACKNOWLEDGMENTS

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FERTILIZATION OF THE MOUSE EGG AND THE EFFECT OF DELAYED COITUS AND OF HOT-SHOCK TREATMENT

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Summary

A total of 329 mated adult mice was used in the investigations.

The mean number of eggs per mouse recovered from 171 mice killed 3-15 hr after ovulation was 8·10 (S.D. 1·94).

In mice maintained under conditions of controlled illumination (light from 3 to 5 a.m., dark from 5 a.m. to 3 p.m.) ovulation began about 11 a.m. and was virtually complete by 4 p.m. The average interval between the ovulation and sperm penetration of an egg was about 5 hr. The sperms spent a mean time of about 35 min in the perivitelline space before entering the vitellus; after a further mean interval of about 2¾ hr the early pronuclei were seen. The mean delay between the beginning of ovulation and the beginning of sperm penetration, in any one mouse, was 2½-3 hr. The mean time required for the majority ($\geqslant$ ¾) of the eggs in any one mouse to be penetrated by sperms, once penetration had begun, was 3½-4 hr. A mean number of 18·6 sperms per tube was found at the site of fertilization shortly after ovulation.

Mice kept under natural lighting conditions were not permitted coitus until 8-8.45 a.m. Sperm penetration of the eggs began between 9 and 9.30 a.m. Once penetration had begun, the average period required for the penetration by sperms of at least three-quarters of the eggs in any one mouse was about 2 hr. The mean number of sperms at the site of fertilization at 9, 9.30, and 10 a.m. was 6·3, 7·9, and 20·6 sperms per tube, respectively. These findings are discussed in relation to the concept that sperms need to spend a period in the female tract to capacitate them for the penetration of the eggs.

The incidence of polyspermy in mouse eggs was not increased by delaying coitus until 3-7 hr after ovulation.

Hot-shock treatment applied to the eggs of mice 3 hr after mating increased the incidence of polyspermy (dispermy) from 0·3 to 3·8 per cent. and of eggs exhibiting suppression of the second polar body from 0·5 to 12·4 per cent. Several other forms of abnormal fertilization were also observed. It is considered that the triploidy reported in mouse embryos following hot-shock treatment of the eggs probably arose in most instances through suppression of second polar body formation, and only occasionally through polyspermic fertilization.

I. INTRODUCTION

Fischberg and Beatty (1952) subjected mouse eggs to an elevated temperature for a short period at about the time of sperm penetration and found that many of the resultant embryos had a triploid number of chromosomes. They postulated that the hot-shock treatment had inhibited the formation of

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the second polar body, without preventing the completion of the second maturation division. Consequently, the embryos were thought to have a diploid number of maternal chromosomes and a haploid number of paternal chromosomes. In reaching this conclusion, Fischberg and Beatty were guided by results obtained on the eggs of newts by Fankhauser and Godwin (1948) and Fischberg (1948), who showed that hot-shock treatment induced triploidy by inhibiting the formation of the second polar body.

Similar treatment of rat eggs, however, did not support Fischberg and Beatty's hypothesis, for, in this species, the extrusion of the second polar body usually took place (Austin and Braden 1954*a*). Moreover, the increased incidence of triploidy could be adequately explained by the relatively high incidence of polyspermy (dispermy) in the treated eggs, for there is strong evidence (Austin and Braden 1953*a*) that dispermy leads to triploidy in the embryo. These triploid embryos would differ from those resulting from suppression of the second polar body, for they would have one maternal and two paternal sets of chromosomes. However, mouse and rat eggs may well differ in their reactions to hot-shock treatment. To test the validity of Fischberg and Beatty's hypothesis it was therefore necessary to examine mouse eggs in the pronuclear stages after such treatment. The results of such an examination are reported in the present paper; in the main, their conclusion is supported. As a preliminary step, a study of the time relations of ovulation and sperm penetration seemed advisable, because little information on this subject was available in the literature.

If, in rats and rabbits, coitus were not permitted until the time of ovulation, or later, a considerable increase in the incidence of polyspermy resulted (Austin and Braden 1953*a*, 1953*b*). Delayed mating thus offered a convenient means for increasing the incidence of triploid embryos. The effect of this procedure has also been studied in the mouse, and the results are reported here.

II. METHODS

Adult mice of mixed stock from our colony were used throughout the investigations. The arrival of eggs in the fallopian tube was taken to be synchronous with ovulation. The eggs were recovered by dissection of the tubes under normal saline solution, and were examined in the fresh state by phase-contrast microscopy in the manner described by Austin and Smiles (1948).

Mice used for the study of the normal time relationships of ovulation and sperm penetration were kept in a cabinet, in which the period of illumination could be accurately controlled. The same cabinet had been used previously for establishing these time relationships in rats (Austin and Braden 1954*b*). The virgin females were kept in the cabinet for 3 wk before the males were introduced; thereafter the males were allowed to remain in the cages continuously.

In the hot-shock experiments, the mice were kept under natural lighting conditions and were allowed to mate between 8 and 9 a.m. Between 11 a.m.

TABLE 1

NUMBERS OF MICE WITH EGGS AND MEAN NUMBERS OF EGGS; MICE KILLED 6-24 HR AFTER THE ONSET OF DARKNESS

Hour of Killing	Number of Mice				Mean Number of Eggs per Mouse			Number of Fallopian Tubes		
	Killed	With Eggs	With Penetrated Eggs	With $\geqslant \frac{3}{4}$ of Eggs Penetrated	Ovulated	Penetrated	With Pronuclei	With Eggs	With Penetrated Eggs	With $\geqslant \frac{2}{3}$ of Eggs Penetrated
11 a.m.	20	3	0	0	0·80	0	0	5	0	0
Noon	20	7	1	0	2·80	0·25	0	14	2	0
1 p.m.	20	12	2	0	4·85	0·25	0	24	3	1
2 p.m.	20	15	3	0	5·65	0·35	0·25	29	4	2
3 p.m.	20	16	7	2	7·15	1·75	0·25	31	10	8
4 p.m.	20	18	13	2	8·10	2·45	0·60	36	20	10
5 p.m.	20	19	16	2	7·85	2·55	0·20	37	26	9
7 p.m.	20	19	15	8	7·55	4·05	1·35	36	27	17
10 p.m.	20	20	19	15	7·90	6·50	4·05	39	36	33
Next day	20	20	20	19	8·05	7·50	—	40	39	39

and noon the animals were anaesthetized by subcutaneous administration of "Nembutal" and incisions were made in the body wall adjacent to the ovaries. The fallopian tubes and ovaries were brought to the exterior and the animal was suspended over a water-bath so that the tubes were completely immersed. The water-bath was maintained at a temperature between 43·5 and 45·0°C and treatment was applied for 5-10 min.

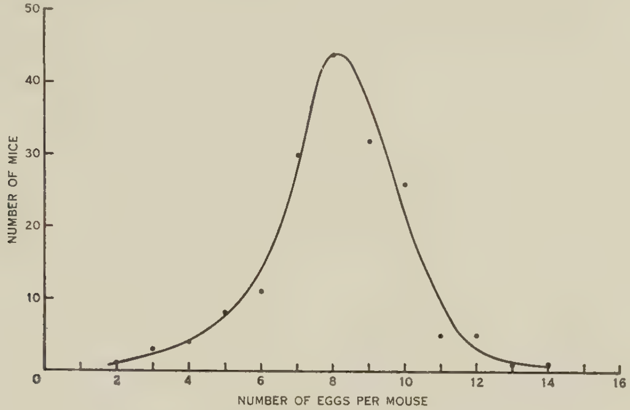

Fig. 1.—Frequency distribution of the number of eggs per mouse recovered from 171 mice.

III. Observations

(a) Number of Eggs Ovulated

Records were kept of the number of eggs found in the fallopian tubes of 171 mice killed 3-15 hr after ovulation. Eggs recovered during this period were usually embedded in the cumulus oophorus, so that it was not difficult to ensure removal of all the eggs present. The frequency distribution of the number of eggs per mouse is shown in Figure 1. The total number of eggs recovered was 1385, and the mean number per mouse 8·10 (S.D. 1·94; range 2-14).

(b) Time Relations of Ovulation and Sperm Penetration

The mice were kept in a cabinet in which the "day" lasted from 3 p.m. till 5 a.m. (solar time) and the "night" from 5 a.m. till 3 p.m. After the males had been introduced, the females were examined at hourly intervals from 10.30 a.m. to 2.30 p.m., and also at 4.30 p.m. In about half of the mice mating occurred before 10.30 a.m.; in most of the remainder it occurred between 10.30 a.m. and 1.30 p.m. In only a small number of mice did mating occur after 1.30 p.m. Twenty mice were killed each hour from 11 a.m. to 5 p.m. and also at 7 p.m., 10 p.m., and during the morning of the following day. The completeness of coitus in each mouse was confirmed by examination of the uterine fluid; if sperms were not found the animal was discarded.

As coitus and ovulation proceeded concurrently in the mice as a group, those killed at the earlier hours could not have been a representative sample

Table 2

NUMBERS OF MICE WITH PENETRATED EGGS AND NUMBERS OF EGGS; MICE KILLED 1-8 HR AFTER MATING AT 8 A.M.

Hour of Killing	Number of Mice			Number of Eggs per Mouse			Number of Fallopian Tubes		
	Killed	With Penetrated Eggs	With $\geqslant \frac{3}{4}$ of Eggs Penetrated	Total	Penetrated	With Pronuclei	With Eggs	With Penetrated Eggs	With $\geqslant \frac{2}{3}$ of Eggs Penetrated
9 a.m.	10	0	0	80	0	0	20	0	0
9.30 a.m.	10	4	0	81	5	0	20	4	0
10 a.m.	10	5	1	83	18	0	20	10	3
11 a.m.	10	7	1	83	23	0	20	11	3
Noon	10	7	4	85	50	10	19	13	12
1 p.m.	10	8	8	77	56	11	20	16	15
4.30 p.m.	15	15	15	104	102	95	30	30	30

of the whole population. For instance, those killed at 11 a.m. of necessity only comprised mice that had mated before 10.30 a.m. If a correlation existed between the time of mating and the time of ovulation in each mouse, then those killed at 11 a.m. would have been "early ovulaters," and the result reported for that hour would be relatively high. The number of mice that mated before 10.30 a.m. was a little less than half the number that were killed at later hours, so that the figures obtained for the hours subsequent to 12 noon may be slightly low.

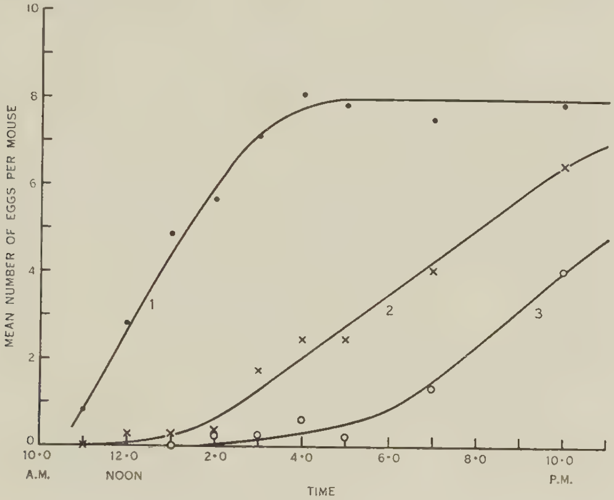

Fig. 2.—Curves illustrating the progressive increase in mean number of eggs per mouse (curve 1), mean number of eggs penetrated by sperms per mouse (curve 2), and mean number of eggs with pronuclei per mouse (curve 3). Mice kept under artificial diurnal light cycle.

The results are given in Table 1 and illustrated graphically in Figures 2 and 3. Ovulation began in the mice as a group just before 11 a.m. and was virtually completed by 4 p.m. One of the mice killed at 5 p.m. and one at 7 p.m. had no eggs in the tubes, but it is not certain that they would have ovulated that day. Lewis and Wright (1935) noted that, in mice, mating occasionally occurred in the absence of ovulation. Curve 1 (Fig. 2) illustrates the progress of ovulation in the mice as a group. Penetration of the eggs by sperms began about midday and reached completion soon after 10 p.m. The progressive increase in the mean number of penetrated eggs per mouse is illustrated in curve 2 (Fig. 2). The average interval between curves 1 and 2 corresponds to about 5 hr, or, in other words, the mean period that elapsed between the ovulation and the penetration of an egg was about 5 hr.

The presence or absence of pronuclei was also noted in penetrated eggs. Pronuclei were first seen at 2 p.m. and the incidence increased progressively up to 10 p.m.; this is illustrated in curve 3 (Fig. 2). The average distance between curves 2 and 3 represents about 3¼ hr, indicating that a mean period of about 3-3½ hr elapsed between sperm penetration of the zona pellucida and formation of pronuclei.

The data (Table 1) can also be used to estimate these time intervals as mean values for any one mouse. This was done by constructing curves relating time with the number of mice containing eggs (or penetrated eggs) as a percentage of the total number of mice killed at each hour. Curve *A* (Fig. 3) represents the percentage of mice found to have eggs in the tubes, and curve *B* the percentage of mice containing one or more penetrated eggs. The average distance between them corresponds to about 2¾ hr, so that the mean interval, for any one mouse, between the beginning of ovulation and the beginning of penetration may be taken as 2½-3 hr. Curve *C* represents the percentage of mice in which at least 75 per cent. of the eggs had been penetrated. The figure of 75 per cent. was chosen because, whereas 7 out of 20 mice killed about 24 hr after mating had less than 100 per cent. of the eggs penetrated, in only one mouse were less than three-quarters of the eggs penetrated. The average distance between curves *B* and *C* corresponds to the average period required, in any one mouse, for the majority of the eggs to be penetrated by sperms, once penetration had begun. It was found to be about 3¾ hr.

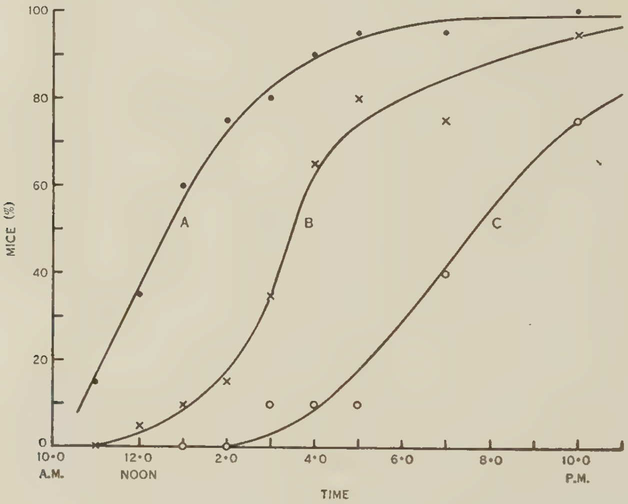

Fig. 3.—Curves showing increase in the percentage of mice with eggs (curve *A*), percentage of mice with one or more penetrated eggs (curve *B*), and percentage of mice that had at least three-quarters of the eggs penetrated by sperms (curve *C*). Mice kept under artificial diurnal light cycle.

In a similar way, the data given in Table 1 can be used to show that the average period required for the penetration of at least two-thirds of the eggs in any one fallopian tube, once penetration had begun, was about 2 hr. The value of two-thirds was taken because in only one out of 40 tubes from mice killed 24 hr after mating were less than two-thirds of the eggs penetrated.

When the eggs were being examined, account was taken of the number of eggs that had a sperm in the perivitelline space, but not in the vitellus. The number of such eggs and the proportion they represented of the total number of eggs recovered at each hour were: noon, 2 eggs (3·6 per cent.); 1 p.m., 2 eggs (2·1 per cent.); 2 p.m., 0 eggs; 3 p.m., 4 eggs (2·8 per cent.); 4 p.m., 7 eggs (4·3 per cent.); 5 p.m., 13 eggs (8·3 per cent.); 7 p.m., 11 eggs (7·3 per cent.); 10 p.m., 9 eggs (5·7 per cent.). As all the penetrated eggs recovered 24 hr after mating contained a sperm in the vitellus, it may be assumed that the eggs seen earlier with sperms only in the perivitelline space would eventually each have a sperm in the vitellus. From curve 2 (Fig. 2) it can be seen that the rate of increase in the number of penetrated eggs was approximately constant over the period 2 to 7 p.m., so that the frequency with which eggs were seen with sperms in the perivitelline space, but not in the vitellus, should be proportional, in this period, to the average time a sperm spent at that site before entering the vitellus. The mean interval was calculated from the foregoing data by the method previously used for the rat (Austin and Braden 1954*b*) and it was found to be approx. 35 min.

Two of the penetrated eggs recovered were abnormal. The first was obviously degenerate; there were nine sperms in the vitellus and eight of them were very active, causing considerable disturbance of the cytoplasmic granules. The heads of the sperms did not exhibit any loss of optical density, such as normally occurs after entry into the vitellus. The second egg contained one male and two female pronuclei. After the egg had been fixed it was seen that from the vicinity of each of the two female pronuclei spindle fibres passed to two polar bodies. The "central bodies" of the second meiotic spindles (Braden and Austin 1954*a*) were also readily distinguishable. The evidence thus seems to establish conclusively that this egg had been a binuclear oocyte.

(*c*) *Effect of Delaying Coitus*

In three groups of mice that had been kept under natural lighting conditions, coitus was not permitted until 8, 9.30, or 11.30 a.m. Males were placed in the cages at those times and removed about 45 min later. Mating did not readily occur at 11.30 a.m. Seventy-five mice that had mated between 8 and 8.45 a.m. were killed at 9, 9.30, 10, 11 a.m., noon, 1 p.m., or 4.30 p.m. (Table 2). Sperm penetration of the eggs in these mice began between 9 and 9.30 a.m. and reached completion in about 6 hr (curve 1, Fig. 4). The mean period required for the penetration of the majority (⩾ ¾) of the eggs in any one mouse was determined by the method employed in the previous section. It was found to be about 1½ hr; the mean period necessary for the penetration of at least two-thirds of the eggs in any one tube was about 1 hr.

Pronuclei were first seen at midday. The mean delay between sperm penetration of the zona pellucida and formation of the pronuclei was about 2½ hr (curve 2, Fig. 4).

No egg was found with more than one sperm in the vitellus. Three eggs recovered at 4.30 p.m. were abnormal; they contained only one pronucleus although a sperm mid-piece was present in the vitellus.

Twenty mice that had mated between 9.30 and 10.15 a.m. were killed at about 4.30 p.m. the same day. Again, no instance of polyspermy was seen, but there were two eggs that had one large (presumably male) pronucleus and two small (presumably female) pronuclei. Neither egg showed evidence of having extruded the second polar body.

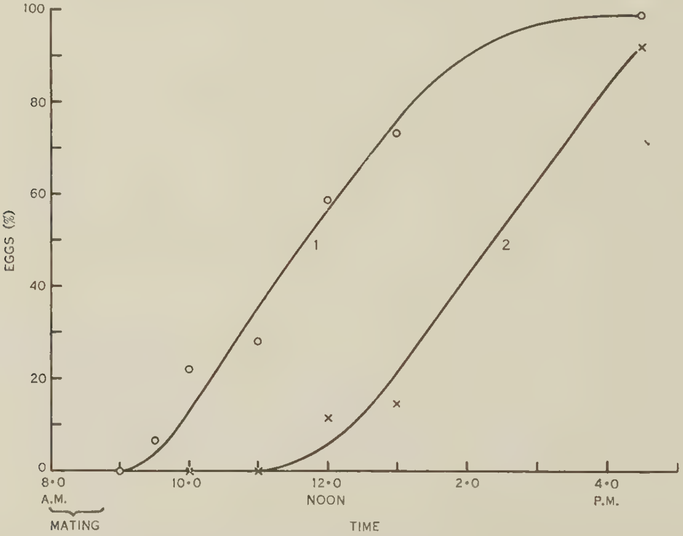

Fig. 4.—Percentage of eggs penetrated by sperms (curve 1) or with pronuclei (curve 2) in mice mated between 8 and 8.45 a.m. Mice kept under natural lighting conditions.

Sixteen mice that had mated between 11.30 a.m. and 12.15 p.m. were killed at about 5 p.m. the same day. One egg was found to be undergoing polyspermic fertilization: two sperm mid-pieces were present in the vitellus, but the pronuclei had not yet formed.

The frequency distribution of eggs containing various numbers of sperms following delayed matings is given in Table 3. Apart from the fact that there were more unfertilized eggs after 11.30 a.m. mating, the distributions do not differ significantly from that reported earlier for eggs recovered after mating at the normal time (Braden, Austin, and David 1954).

(*d*) *Number of Sperms at the Site of Fertilization*

In some of the mice kept under conditions of controlled illumination, the number of sperms at the site of fertilization was counted, using Austin's (1952) method. Estimates were made on six mice killed at midday, one at 1 p.m., one at 2 p.m., five at 3 p.m., three at 4 p.m., and three at 5 p.m. Although the mean number of sperms per ampulla was higher at the later times, the increase was not statistically significant. The mean number of sperms for all the mice was 18·6 and the standard error of the mean was 2·43 (range 1-51).

In the mice kept under natural lighting conditions and mated between 8 and 8.45 a.m., the number of sperms present at the site of fertilization was determined at 9, 9.30, and 10 a.m. The mean values with their standard errors were: 9 a.m. (10 mice) 6·3 ± 2·2, 9.30 a.m. (8 mice) 7·9 ± 2·4, and 10 a.m. (5 mice) 20·6 ± 6·0. No sperms were found in 10 out of the 20 tubes from mice killed at 9 a.m., and in one tube from those killed at 9.30 a.m.

(*e*) *Effect of Hot-shock Treatment*

The eggs of 18 mice which had been kept under natural lighting conditions and mated between 8 and 9 a.m. were subjected to hot-shock treatment for 5-10 min between 11 a.m. and noon. The animals were killed 4-5 hr later and a total of 132 eggs was recovered. Of these, 27 eggs were unpenetrated and 37 others contained sperms but were fragmented. Twelve of the 27 unpenetrated eggs contained a large single nucleus resembling a normal pronucleus; this phenomenon has been reported elsewhere (Braden and Austin 1954*a*). Of the remaining 68 penetrated eggs, only 37 could be classified as normal: they each contained two apparently normal pronuclei and one sperm mid-piece in the vitellus; in addition, one well-formed and one more or less disintegrated polar body were visible in most eggs. The abnormalities of fertilization seen among the remaining 31 eggs included:

(1) Four eggs that were polyspermic (dispermic): they each had one small and two large pronuclei and two sperm mid-pieces in the vitellus.

(2) Thirteen eggs that each contained one large and two small pronuclei and one sperm mid-piece. The two small pronuclei were usually identical in size and form. Only one small, rather degenerate, polar body was seen in most of these eggs; in the remainder no polar bodies could be detected.

(3) Four eggs in which there was one normal pronucleus and one to three subnuclei.

(4) Five eggs in which there was only a single large pronucleus. In three of the eggs, the pronucleus, because of its proximity to the sperm mid-piece, seemed to be the male. In the other two eggs the male element appeared to be represented by a small vacuole lying between the mid-piece and acrosome. These two types of abnormality may possibly represent early phases of androgenesis and gynogenesis, respectively.

(5) In one egg, four sperm mid-pieces and four acrosomes were visible in the vitellus, but only a single pronucleus had formed. The sperm nuclei appeared as small spherical vacuoles, a stage which appears to occur in normal eggs just prior to pronucleus formation.

(6) Four 2-cell eggs; two of the eggs had one mononucleate and one binucleate blastomere similar to that illustrated in an earlier paper (Austin

and Braden 1954*c*). The other two eggs closely resembled normal 2-cell eggs. One sperm mid-piece was present in each of the four eggs, but well-formed polar bodies were not seen. The most logical explanation of the mode of origin of these eggs, found in the 2-cell stage so soon after sperm penetration, is that they were formed by a process similar to that which apparently occurs in heat-treated unfertilized eggs and which has been referred to as "immediate cleavage" (Braden and Austin 1954*a*). One of the two nuclei in the binucleate blastomeres could therefore have been a male pronucleus.

TABLE 3

FREQUENCY DISTRIBUTION OF EGGS CONTAINING VARIOUS NUMBERS OF SPERMS; EGGS FROM MICE KEPT UNDER NORMAL LIVING CONDITIONS

Time of Mating	Number of Mice	Total Eggs	Unpenetrated Eggs	Number of Eggs Penetrated by *i* Sperms							Total Penetrated Eggs
				i= 1	2	3	4	5	6	⩾7	
Normal*	20	178	9	138	27	2	1	0	1	0	169
8 a.m.†	15	104	2	84	11	4	2	1	0	0	102
9.30 a.m.	20	170	3	126	29	5	6	1	0	0	167
11.30 a.m.	16	130	24	77	27	2	0	0	0	0	106
8 a.m.—Hot shock	18	135	26	66	28	3	3	2	3	4 (8, 9, 13, 16)	109

* These figures have been reported previously (Braden, Austin, and David 1954).

† Mice killed at 4.30 p.m. only.

The frequency distribution of eggs containing various numbers of sperms is given in Table 3. Excluding the unpenetrated eggs, the proportion of eggs that contained more than one sperm was significantly greater ($P < 0{\cdot}001$) in the heat-treated mice than in the untreated mice that had been mated at the same time (8 a.m.). This indicates that the normal reaction of the zona pellucida to sperm penetration (Braden, Austin, and David 1954) had been partially inhibited by the hot-shock treatment.

IV. DISCUSSION

It has been reported elsewhere (Austin and Braden 1954*b*) that when rats were kept under natural lighting conditions (14-hr day), ovulation began in the rats as a group about midnight (i.e. 5-6 hr after sunset), and was complete by 4 a.m. When the times of light and dark were artificially reversed, the time of ovulation was also changed: it still began about 6 hr after the onset of darkness and reached completion in about 4 hr. The available evidence indicates that, in mice also, ovulation is regulated by the times of light and dark. Snell *et al.* (1940) and Snell, Hummel, and Abelmann (1944) showed that oestrus began in mice 3-6 hr after the onset of darkness, irrespective of the solar time, and ovulation followed 1-4 hr later. When the period of darkness began at 6.15 a.m. each day ovulation commenced in the mice as a group about 5 hr later, and reached completion in about 5½ hr. These findings are in close agreement with those reported in the present paper.

In mice and rats subjected to identical diurnal light cycles the time relationships of ovulation and sperm penetration were found to be very similar. As already noted, the interval between the onset of darkness and the beginning of ovulation in the animals as a group was 5-6 hr for both species. For other time relationships, the mean values obtained for rats (Austin and Braden 1954*b*) were a little shorter than those found for mice. For instance, the period required for the completion of ovulation in the animals as a group was about 4 hr for rats and about 5 hr for mice, and the average interval between ovulation and the penetration of an egg in rats was 3 hr and in mice 5 hr. These differences appear to be referable, in part at least, to greater variability between animals in the mice.

In rats mated after the time of ovulation it has been found that sperm penetration does not begin until 2-4 hr after coitus, even though sperms are present about the eggs at 1 hr (Austin 1952; Austin and Braden 1954*b*; Braden and Austin 1954*b*). This was taken as support for the concept that a sperm must spend a certain period in the female genital tract for "capacitation" before it is able to penetrate the egg (Austin 1951; Chang 1951; Austin and Braden 1952). The present data indicate that, if this is true in mice also, the period required for "capacitation" must be much shorter than in rats, for, when mating occurred between 8 and 8.45 a.m., sperm penetration began at about 9.30 a.m. The lower limit of the capacitation period, which in rats is 2 hr, is therefore possibly about 1 hr in mice. As the mean number of sperms at the site of fertilization was small at 9 a.m. and had increased considerably by 10 a.m., the delay of about 1 hr between coitus and the beginning of sperm penetration of the mouse eggs may represent simply the period required for sperms to ascend the genital tract and to meet the eggs. It does not seem possible to decide between these two alternative explanations on the data available at present.

Lewis and Wright (1935) found sperms at the site of fertilization 15 min after coitus in one mouse and 40 min after coitus in another. Merton (1939) reported sperms about the eggs in three mice killed 2, 2½, and 3 hr after artificial insemination at the first post-partum oestrus. In three others killed at ½, 1, and 1½ hr after insemination he found no sperms in the ampullae. In the present investigations, sperms were found at the site of fertilization in 10 out of 20 tubes from animals mated between 8 and 8.45 a.m. and killed at 9 a.m. At 9.30 a.m. 15 out of the 16 tubes examined had sperms in the ampullae. The mean number of sperms per ampulla rose from 6·3 at 9 a.m. to 20·6 at 10 a.m. From the foregoing data it may be concluded that, in the mouse, sperms may reach the site of fertilization within 15 min after coitus, but that they are not present at this location in the majority of animals until about 1 hr after coitus; the mean period required for the ascent of sperms to the site of fertilization is possibly about ¾ hr.

Several workers have published estimates for the duration of the interval in mice between coitus or artificial insemination and sperm penetration of the eggs. Comparison is difficult, however, because, in three instances, the first post-partum oestrus was used; the time relations of oestrus, ovulation, sperm transport, and the penetration of the eggs may well be different under such circumstances. The estimates given were 6-10 hr (Gerlach 1906), 4-7 hr (Long and Mark 1911), and 3-4 hr (Merton 1939). Mating at the second oestrus post-partum was employed by Sobotta (1895), whereas Lewis and Wright (1935) and the present authors used virgin females. Sobotta's estimate of the interval was 6-10 hr, based on the results from one animal; Lewis and Wright saw sperms in the perivitelline space of the eggs of one mouse as early as 2 hr after coitus. In the present series, the mean interval between ovulation and sperm penetration was approximately 5 hr, and coitus generally took place 1-4 hr prior to ovulation, so that the mean interval between coitus and sperm penetration may be taken as 7-8 hr. The shorter interval reported by Lewis and Wright (1935) occurred in an animal that was not allowed coitus until it was in "mid-oestrus," as judged by the vaginal smear. Ovulation had probably already taken place, and this may explain the short interval, for, in the present series, it was found that, if mating was delayed until 3-8 hr after ovulation, the interval between coitus and sperm penetration was 1-5 hr. It seems that penetration of the eggs by sperms proceeds more quickly when coitus is delayed until after ovulation. A similar phenomenon was noted in the rat (Austin and Braden 1954*b*) and it was suggested that maturation of the egg membranes after ovulation was necessary before sperm penetration could occur.

Long and Mark (1911) thought that the pronuclei were formed within a few minutes of sperm penetration in the mouse; however, the present observations show that this is not so. Sobotta (1895) concluded that the interval required was of the order of 1 hr, but even this estimate appears to be low. It has now been found that sperms spend, on the average, about ½ hr in the perivitelline space before entering the vitellus, and that a further period of 2-3 hr is required for the formation of the pronuclei. These estimates are similar to those derived for the rat (Austin and Braden 1954*b*).

The incidence of polyspermy in rat eggs was shown to be greatly increased by delaying coitus until the time of ovulation or later (Austin and Braden 1953*a*, 1953*b*), but this has not been found to be so for mice. From 20 mice mated at the normal time two polyspermic eggs were recovered (Braden, Austin, and David 1954), whereas, in the present series, only one instance of the phenomenon was seen amongst the eggs from 51 mice mated 3-12 hr after ovulation. Hot-shock treatment, on the other hand, caused a considerable increase in the incidence of polyspermy in the eggs of both species. Suppression of second polar body formation in penetrated eggs was also seen much more commonly after hot-shock treatment than in untreated animals. In rat eggs the effect was transient; the initial disturbance of the correlation in the development of male and female elements was soon adjusted and fertilization then proceeded normally (Austin and Braden 1954*a*). In mouse eggs, however, it appears that the second meiotic division was completed, but both groups of chromosomes remained within the egg and formed pronuclei, so that one male and two female pronuclei were formed. Two female pronuclei have been shown to originate in this manner in *Triturus* eggs, following hot-shock treatment (Fankhauser and Godwin 1948).

Hot-shock treatment of mouse eggs at about the time of sperm penetration has been shown by Fischberg and Beatty (1952) to result in a quite high incidence of triploidy in 3½-day embryos. They thought it very likely that the condition arose by suppression of second polar body formation, the embryos thus having one paternal and two maternal sets of chromosomes. The present findings clearly support this conclusion. In some (about 25 per cent.) of the mouse embryos, however, the triploidy may well have arisen from polyspermic (dispermic) fertilization, and this would have resulted in embryos with one maternal and two paternal sets of chromosomes.

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PRESERVATION OF RABBIT, SHEEP, AND OX RED CELLS AT −79°C

By A. W. Blackshaw*

[*Manuscript received April 26, 1954*]

Summary

Observations made on the freezing of red cells at −79°C show that equilibration of the cells with a glycerol diluent reduces the amount of haemolysis in rabbit blood but increases it in sheep and ox blood. When ethylene glycol is substituted for glycerol, no effect of equilibration is detectable in rabbit blood, but haemolysis is increased in sheep and ox blood.

The addition of arabinose to the glycerol or ethylene glycol diluents reduces haemolysis after freezing with sheep and ox blood, but on rabbit blood it has no effect with glycerol and increases the haemolysis when used with ethylene glycol.

Possible modes of action of these substances are discussed.

I. Introduction

The recent observations of Smith (1950) and Sloviter (1951) have shown that rabbit and human red cells may be satisfactorily preserved for long periods at −79°C without appreciable haemolysis, by the prior dilution of the blood with a medium containing glycerol. Other methods have been successful in preserving red cells, but very special conditions were necessary; the use of thin films and rapid freezing in liquid nitrogen (Luyet 1949) or rigid temperature control at −3°C (Strumia 1949).

To achieve best results with methods using glycerol the following conditions had to be fulfilled (Sloviter 1951):

(i) A final concentration of glycerol between 10 and 15 per cent.

(ii) Rapid freezing and thawing.

(iii) The equilibration with glycerol for at least 1 hr before freezing. Failure to equilibrate caused considerable haemolysis. This process, in which the cells are suspended in a glycerol diluent for a period of one or more hours before freezing is to allow time for the glycerol to completely permeate the cells and so exert its protective action.

The presence of sugars, in particular sucrose and lactose at 3-5 per cent. concentrations, has been of value in preserving red cells at −3°C (Strumia *et al.* 1950). The addition of pentose and other sugars to glycerol media used for freezing bull semen has increased revival and also made possible good revival of ram semen (Emmens and Blackshaw 1950).

In view of these observations and to extend the range of animal species examined, studies have been made on the effect of freezing red cells of the rabbit, sheep, and ox in diluents containing various combinations of glycerol and arabinose, which is a readily available pentose sugar, and in addition ethylene glycol.

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II. Materials and Methods

Blood from rabbits, sheep, and cattle was taken by veni-puncture, 0·38 per cent. sodium citrate being added to prevent clotting. The blood was either used the same day or stored overnight at 5°C and used the following day. No attempt was made to obtain sterile blood. Diluents, which were tested for their effect on red cells on freezing, were 30 per cent. glycerol, 30 per cent. ethylene glycol (v/v) in 0·8 per cent. sodium chloride, and the saline solution itself. When required, arabinose (Gurr) was added to give 5 per cent. (w/v) concentration. Before freezing, equal volumes of blood and diluent were carefully mixed, the final concentrations of glycols being 15 per cent. and of arabinose 2½ per cent.

Table 1

INFLUENCE OF GLYCEROL, ARABINOSE, AND EQUILIBRATION ON THE HAEMOLYSIS OF RABBIT RED CELLS AFTER FREEZING TO –79°C

Optical densities

Individuals	Treatments												
	Equilibrated						Not Equilibrated						Totals
Diluents	1	2	3	4	5	Sum	1	2	3	4	5	Sum	
Glycerol	0·27	0·06	0·07	0·05	0·16	0·61	0·47	0·30	0·16	0·20	0·38	1·51	2·12
Glycerol and arabinose	0·19	0·06	0·08	0·05	0·28	0·66	0·36	0·30	0·19	0·12	0·39	1·36	2·02
Saline	0·77	1·00	1·20	0·53	0·95	4·45	0·80	1·00	0·95	0·48	1·00	4·23	8·68
Saline and arabinose	0·64	0·85	0·95	0·35	0·95	3·74	0·62	0·85	0·95	0·42	1·00	3·84	7·58
Totals	1·87	1·97	2·30	0·98	2·34	9·46	2·25	2·45	2·25	1·22	2·77	10·94	20·40

Before mixing, the blood and diluents were cooled to 5°C. Immediately after mixing 1 ml amounts of diluted blood were placed in 6 by ½ in. soda-glass test-tubes and rapidly frozen to –79°C. One hour after mixing, to allow equilibration of the cells with glycerol, further 1 ml samples were taken and rapidly frozen to –79°C. All frozen samples were kept at –79° for 1 hr and then rapidly thawed in a water-bath at 37°C. Control samples of blood were taken at the same time as those to be frozen and the plasma separated by centrifuging. After thawing, the plasma was also removed from previously frozen specimens.

To estimate cell damage, the optical density of supernatant plasma diluted 1 in 50 with 0·8 per cent. sodium chloride was measured. Measurements were made with a Coleman spectrophotometer at 550 mμ with a suitable filter. A reference solution for each blood specimen was prepared from supernatants of the unfrozen, non-equilibrated tubes diluted with 0·8 per cent. sodium chloride only.

Analyses of variance of the results were performed, using the measurements of optical density as unit variable. The relation between optical density (D) and percentage transmittance (T) is given by the formula $D = -\ln T$, and the higher the density, the greater the cell damage.

Table 2

INFLUENCE OF ETHYLENE GLYCOL, ARABINOSE, AND EQUILIBRATION ON THE HAEMOLYSIS OF RABBIT RED CELLS AFTER FREEZING TO −79°C

Optical densities

Individuals	Treatments														
	Equilibrated							Not Equilibrated							Totals
Diluents	1	2	3	4	5	6	Sum	1	2	3	4	5	6	Sum	
Ethylene glycol	0·03	0·08	0·06	0·04	0·06	0·11	0·38	0·03	0·07	0·06	0·04	0·26	0·18	0·64	1·02
Ethylene glycol and arabinose	0·09	0·10	0·05	0·34	0·22	0·05	0·85	0·26	0·25	0·08	0·21	0·23	0·12	1·15	2·00
Saline	0·85	0·67	0·64	0·83	0·95	1·00	4·94	0·93	0·64	0·66	0·85	0·95	1·00	5·03	9·97
Saline and arabinose	0·85	0·66	0·78	0·93	0·95	1·00	5·17	0·90	0·64	0·62	0·93	0·95	1·00	5·04	10·21
Totals	1·82	1·51	1·53	2·14	2·18	2·16	11·34	2·12	1·60	1·42	2·03	2·39	2·30	11·86	23·20

III. RESULTS

Two separate factorial experiments were done with rabbit blood. In the first, five replications, each on a different blood sample, were used to test the effect of glycerol, arabinose, and equilibration on the survival of red cells after freezing (Table 1), and in the second experiment, with six replications, ethylene glycol was substituted for glycerol (Table 2). Summary analyses of variance, omitting sums of squares and mean squares, are given in Table 3, for both sets of data. Haemolysis in the control tubes was slight and no values are included in the tables.

Analysis of the first experiment shows that equilibration of the cells before freezing helps to prevent haemolysis, but the effect is limited to those suspended in glycerol. The addition of arabinose to the medium has no significant effect on the amount of haemolysis. The significant sample-glycerol interaction indicates the varying effectiveness of the glycerol as a protective agent.

The situation with ethylene glycol is quite different; equilibration does not influence results and arabinose significantly increases the amount of haemolysis produced by freezing. The results for saline and saline-arabinose for both experiments were combined into equilibrated and non-equilibrated groups and the two diluents compared within each group, using the *t*-test. In neither case was there any significant difference between the diluents.

TABLE 3

SUMMARY ANALYSES OF VARIANCE FOR THE DATA OF TABLES 1 AND 2

Sources of Variation	Glycerol		Ethylene Glycol	
	Degrees of Freedom	Variance Ratio	Degrees of Freedom	Variance Ratio
1. Blood samples	4	46·7*	5	17·3*
2. Equilibration	1	18·9*	1	—
3. Arabinose	1	1·0	1	9·1*
4. Glycerol or ethylene glycol	1	1112·8*	1	1804·3*
Interactions:				
2/3	1	—	1	—
2/4	1	22·3*	1	2·2
3/4	1	—	1	3·3
2/3/4	1	2·0	1	—
1/2	4	1·1	5	1·0
1/3	4	4·4**	5	2·3
1/4	4	41·3*	5	9·5*
Residual mean squares	16	0·0033	20	0·0034

* $P<0{\cdot}01$.

** $P<0{\cdot}05$.

The four replications with ox blood and five with sheep blood are listed in Tables 4 and 5. Ethylene glycol has been included in these tests but the design is not factorial. Summary analyses of variance for both species are given in Table 6 and show that for both species equilibration is not only unnecessary but also harmful to red cell survival. On the other hand arabinose has a highly significant beneficial effect with both species, although the significant equilibration-arabinose interaction with ox blood indicates that the effect is limited to the equilibrated specimens.

The use of ethylene glycol is superior to glycerol when freezing ox blood but with sheep blood the significant ethylene glycol interaction, with equilibration shows that only after this treatment is it better than glycerol.

IV. Discussion

Smith (1950) and Sloviter (1951) have shown that, prior to freezing, a period of equilibration is necessary with a glycerol medium to achieve maximum

Table 4

INFLUENCE OF GLYCEROL, ETHYLENE GLYCOL, ARABINOSE, AND EQUILIBRATION ON THE HAEMOLYSIS OF OX RED CELLS AFTER FREEZING TO −79°C

Optical densities

Individuals	Treatments										
	Equilibrated					Not Equilibrated					Totals
Diluents	1	2	3	4	Sum	1	2	3	4	Sum	
Glycerol	0·40	0·28	0·51	0·57	1·76	0·06	0·13	0·27	0·24	0·70	2·46
Glycerol and arabinose	0·24	0·17	0·36	0·48	1·25	0·07	0·09	0·24	0·34	0·74	1·99
Ethylene glycol	0·15	0·25	0·62	0·33	1·35	0·04	0·06	0·07	0·14	0·31	1·66
Ethylene glycol and arabinose	0·05	0·08	0·08	0·09	0·30	0·06	0·05	0·03	0·07	0·21	0·51
Saline	0·68	0·85	1·10	1·10	3·73	0·46	0·93	1·10	1·10	3·59	7·32
Saline and arabinose	0·51	0·76	0·90	0·85	3·02	0·57	0·85	0·90	0·95	3·27	6·29
Totals	2·03	2·39	3·57	3·42	11·41	1·26	2·11	2·61	2·84	8·82	20·23

Table 5

INFLUENCE OF GLYCEROL, ETHYLENE GLYCOL, ARABINOSE, AND EQUILIBRATION ON THE HAEMOLYSIS OF SHEEP RED CELLS AFTER FREEZING TO −79°C

Optical densities

Individuals	Treatments												
	Equilibrated						Not Equilibrated						Totals
Diluents	1	2	3	4	5	Sum	1	2	3	4	5	Sum	
Glycerol	0·41	0·25	0·77	0·60	0·61	2·64	0·10	0·09	0·22	0·11	0·24	0·76	3·40
Glycerol and arabinose	0·39	0·34	0·47	0·38	0·47	2·05	0·11	0·15	0·20	0·04	0·13	0·63	2·68
Ethylene glycol	0·04	0·41	0·22	0·04	0·48	1·19	0·04	0·28	0·19	0·00	0·17	0·68	1·87
Ethylene glycol and arabinose	0·01	0·30	0·04	0·04	0·09	0·48	0·04	0·06	0·05	0·18	0·06	0·39	0·87
Saline	0·76	0·93	0·83	0·87	1·00	4·39	0·75	0·95	0·85	0·87	1·20	4·62	9·01
Saline and arabinose	0·72	0·72	0·83	0·76	0·78	3·81	0·68	0·83	0·90	0·79	0·75	3·95	7·76
Totals	2·33	2·95	3·16	2·69	3·43	14·56	1·72	2·36	2·41	1·99	2·55	11·03	25·59

recovery of rabbit and human red cells, which seems to indicate that penetration of glycerol into the cell might be essential for its preservation. In support of this, Lovelock (1953*a*) suggests that the main source of damage associated with freezing is the high concentration of electrolytes both inside and outside the cell, and that damage is to the cell as a whole and not merely to the surface structure in direct contact with the medium, and that glycerol lessens the rise in salt concentration on freezing so that if it is prevented from permeating the human red cell destruction occurs, even though the salt concentration outside does not increase unduly. However, the red cells of the ox and sheep are only slightly permeable to glycerol, in contrast to those of man and the rabbit, which are unusually permeable (Jacobs 1931), and equilibration is not only unnecessary but also harmful. However, ethylene glycol penetrates all these cells fairly rapidly, at about the same rate as glycerol with man and the rabbit (Jacobs, Glassman, and Parpart 1935), but in those examined, equilibration is also unnecessary, although not harmful.

TABLE 6

SUMMARY OF RESULTS OF ANALYSES OF VARIANCE FOR DATA OF TABLES 4 AND 5

Sources of Variation	Ox		Sheep	
	Degrees of Freedom	Variance Ratio	Degrees of Freedom	Variance Ratio
1. Blood samples	3	33·1*	4	5·1*
2. Equilibration	1	27·1*	1	22·1*
3. Arabinose	1	28·3*	1	15·6*
4. Diluents	2	444·1*	2	285·2*
a. Glycol *v*. ethylene glycol	1	31·1*	1	29·7*
b. Glycerol-ethylene glycol *v*. saline	1	856·7*	1	541·7*
Interactions:				
1/2	3	1·36	4	—
1/3	3	2·23	4	2·1
1/4	6	6·9*	8	2·0
2/3	1	14·4*	1	1·1
2/4*a*	1	1·9	1	19·4*
2/4*b*	1	17·2*	1	19·1*
3/4*a*	1	2·7	1	—
3/4*b*	1	—	1	—
Residual variance	23	0·0052	28	0·0094

*$P < 0{\cdot}01$.

From these results it seems possible that the protective action of glycerol and ethylene glycol for red cells is not dependent on penetration into the cell. All of the protective action of glycerol for two species could be related to physical changes in the medium or in the cell membrane, which Lovelock (1953*a*, 1953*b*) finds unable to prevent haemolysis in the absence of cell penetration. Smith, Polge, and Smiles (1951) observe that ice crystal formation is not prevented in the presence of glycerol, but the crystal shape is altered and the distance between crystals is increased.

Preliminary dilatometric observations have indicated that in glycerol-water solutions frozen to −20°C, considerable amounts of water do not form ice, the amount bound per g of glycerol being up to 2 g (unpublished data). If Lovelock's suggestion of high salt concentrations being the main cause of haemolysis on freezing is correct, it is clear that if glycerol prevents water from forming ice, the salt concentration cannot rise to haemolytic levels. The part played by arabinose is obscure, particularly as haemolysis is increased when it is used with rabbit blood in the presence of ethylene glycol, but it is known that the red cells of the rabbit, sheep, and ox are not readily penetrated by arabinose (Kozawa 1914). Sucrose and lactose, which do not penetrate human red cells, also help to prevent haemolysis when these cells are frozen to −3°C (Strumia *et al.* 1950) and their action also appears to be associated with changes in the medium or cell surface.

Any direct action of these substances on the cell membrane may be associated with the prevention of protein denaturation or with the breakdown of lipoproteins, which occur in the membrane. Sucrose and glycerol have been shown by Beilinsson (1929) to inhibit heat coagulation of egg albumin, while sucrose and glucose also prevent the coagulation of plant sap proteins by freezing (Hardt, Huddleson, and Ball 1943).

Further observations by Lovelock (1953*b*, 1954) on freezing red cells indicate that the high salt concentrations produced by ice formation do cause dispersion of lipids and lipoproteins from the cell membrane which may be modified by the presence of glycerol.

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THE PREVENTION OF TEMPERATURE SHOCK OF BULL AND RAM SEMEN

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Summary

The decreased vitality of ram and bull spermatozoa caused by sudden cooling (cold shock) may be largely prevented by the presence of egg yolk in the diluent.

The activity of egg yolk in preventing cold shock lies in the alcohol-soluble, acetone-insoluble fraction. The phospholipid lecithin may be isolated from this and will prevent cold shock in concentrations as low as 0·12 per cent.

The protective action of lecithin for ram spermatozoa is greater at pH 6·5 than at a neutral or alkaline pH.

I. Introduction

The collection and storage of semen subjects spermatozoa to numerous environmental changes, which all tend to reduce the number of living and active cells. Sudden changes in temperature affect the vitality of spermatozoa; Milovanov (1934) calls the harmful effect of sudden cooling, "temperature or cold shock." Thus, Birillo and Puhaljskii (1936) observed that a fall in temperature from 40 to 0°C in 30-60 min caused temperature shock to ram and bull spermatozoa. Cooling over 2 hr caused less shock and if it was prolonged to 3 or 4 hr, cold shock did not occur. However, positive results with ram semen were only obtained with artificially buffered specimens. Gladcinova (1937) reported that rapid cooling of horse, bull, and ram semen from body temperature to 5 or 10°C, caused reduced activity which could not be restored by rewarming. Cooling to 20 or 30°C did little harm. It was also noted that temperature-shocked spermatozoa rapidly died at 37°C.

Ram semen was investigated by Gunn, Saunders, and Granger (1942), who found no advantage in slow cooling, but placed the semen in a container at 4°C as soon as collected. A cooling rate of not more than 5°C an hour was recommended by Easley, Mayer, and Bogart (1942) for bull semen, but semen diluted with egg yolk could be cooled rapidly, although sudden cooling to 0°C caused significant motility loss. Earlier, Phillips and Lardy (1940) had recommended the use of egg yolk as a protective agent against cold shock and for the storage and transport of semen.

Epididymal spermatozoa of the bull and boar have been shown to be very resistant to temperature shock, but after ejaculation the cells have little resistance (Lasley and Bogart 1944; Lasley and Mayer 1944). The addition of egg yolk to the semen before cooling restores resistance.

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A factor, which greatly increases the number of spermatozoa resistant to cold shock, has been isolated from egg yolk (Mayer and Lasley 1944). The factor was acetone, alcohol, and ether insoluble, and gave a positive ninhydrin reaction. An acetone-insoluble, alcohol-soluble fraction was toxic, killing the spermatozoa within a few minutes at room temperature. Mayer and Lasley (1945) also showed that ram spermatozoa did not acquire much resistance from the factor; maximum survival after cold shock did not exceed 30 per cent. On the other hand, Walton (1947) observed that the ether-soluble or fatty fraction of egg yolk completely protected bull spermatozoa from cold shock.

Table 1

EFFECT OF EGG YOLK ON THE DEGREE OF COLD SHOCK WITH BULL AND RAM SPERMATOZOA

½ Hr motility scores

Bull Number	Bull				Ram Number	Ram			
	20 Per Cent Egg Yolk Citrate		Buffered Glucose Sodium Chloride			20 Per Cent. Egg Yolk Citrate		Buffered Glucose Sodium Chloride	
	Control	Shocked	Control	Shocked		Control	Shocked	Control	Shocked
1	3·3	3·3	3·5	2·5	1	3·8	3·8	3·3	2·0
2	2·5	2·5	2·8	1·5	2	3·5	3·0	3·5	1·5
3	3·0	2·5	2·8	1·0	3	3·0	2·8	3·3	1·0
4	3·3	3·3	3·5	2·3	4	3·3	3·0	3·3	1·8
5	3·5	3·5	3·5	2·3	5	3·5	3·0	3·5	0·5
6	3·0	3·0	3·0	1·5	6	4·0	3·5	4·0	1·5
7	3·3	3·3	3·0	1·0					
Totals	21·9	21·4	22·1	12·1		21·1	19·1	20·9	8·3
Mean score	3·1	3·0	3·2	1·7		3·5	3·2	3·5	1·4

II. Materials and Methods

Fresh bull and ram ejaculates of good initial motility were used in the tests. Bull semen was collected by means of an artificial vagina and ram semen by electrical ejaculation (Gunn 1936). Precautions were taken to avoid sudden changes in temperature and the ejaculates were used as soon after collection as possible.

The diluents used were (i) 3 per cent. glucose, 0·26 per cent. sodium chloride containing 20 ml of 0·1M phosphate buffer per 100 ml. The usual pH was 7·2; variations are recorded when necessary, (ii) 3 per cent. sodium citrate containing phosphate buffers as above.

A standard procedure was used to produce cold shock. Before dilution, the buffers were brought to the temperature of the semen, usually that of the room, 0·2 ml of semen was added to 2·0 ml of diluent and gently mixed. The suspension was then split into equal parts, one was kept at room temperature, the other rapidly chilled by immersion in a bath of ice and water. After 10 min the chilled specimens were removed and allowed to return to room temperature. Observations of motility were made on the control tubes as soon after mixing as possible. After half an hour both control and chilled tubes were examined for motility. In some experiments observations were made for 2 or 3 hr but this was abandoned in the later tests.

Estimations of motility were made as described by Emmens (1947) in which full activity is scored as four, complete immotility as zero.

TABLE 2

EFFECT OF EGG YOLK AND SOME YOLK CONSTITUENTS ON THE OCCURRENCE OF COLD SHOCK

½ Hr motility scores

Diluent	Bull Ejaculates					Ram Ejaculates						
Control	1	2	3	4	Total	1	2	3	4	5	6	Total
(1) Glucose-sodium chloride	2·5	3·3	3·0	2·5	11·3	3·0	1·5	3·3	3·5	3·3	3·5	18·1
Cold shocked												
(2) Glucose-sodium chloride	1·0	2·5	0·5	0·5	4·5	0·5	1·0	1·0	2·0	1·0	1·0	6·5
(3) 1% Freeze-dried egg yolk	2·3	3·5	2·5	2·0	10·3	2·5	1·5	3·0	2·8	3·0	3·0	15·8
(4) 1% Acetone-insoluble residue of (3)	2·3	3·5	2·5	2·5	10·8	2·5	1·0	2·8	3·0	2·8	3·0	15·1
(5) 0·5% Acetone-soluble extract of (3)	1·0	2·0	0·5	0·5	4·0	1·5	0·0	2·0	3·0	1·8	1·0	9·3
(6) 0·5% Alcohol extract of (4)	2·3	3·0	3·0	2·0	10·3	2·5	1·5	2·8	3·3	3·0	2·8	15·9
(7) 0·5% Acetone precipitate of (6)	2·3	3·5	3·0	2·5	11·3	3·0	2·3	3·0	3·3	3·0	2·8	17·4
(8) 1% Acetone, alcohol, ether-insoluble residue of (3)	1·8	3·0	1·0	0·5	6·3	1·0	1·0	1·3	2·0	1·0	1·3	7·6

The data of Tables 1, 2, 5, and 6 were examined by the analysis of variance and in those cases where a *t*-test was later used, *t* was calculated using the error mean square and the mean of the differences between the treatments being compared. The degrees of freedom for the error mean square were used to determine probability levels.

When *t*-tests alone were used (Tables 3 and 4), *t* was calculated using the mean of the difference between treatment pairs. In the text the degrees of freedom are given in brackets after *t*.

III. RESULTS

Preliminary experiments were made with 20 per cent. egg yolk in sodium citrate diluent to determine the protection afforded bull and ram spermatozoa against cold shock (Table 1). Analyses of the data showed significant treatment effects, $F = 48{\cdot}1$, $DF =$ 3 and 18, $P < 0{\cdot}01$ (bull), and $F = 63{\cdot}9$, $DF = 3$ and 15, $P < 0{\cdot}01$ (ram). The two groups which had been cold shocked were compared using a t-test and $t_{(18)} = 9{\cdot}1$, $P < 0{\cdot}01$ for bull spermatozoa, while $t_{(15)} = 10{\cdot}1$, $P < 0{\cdot}01$ for ram spermatozoa. There were clearly no differences between the control groups and the cold-shocked group containing egg yolk, which shows the efficiency of egg yolk in preventing cold shock.

TABLE 3

EFFECT OF 0·5 PER CENT. CRUDE LECITHIN ON THE DEGREE OF COLD SHOCK

½ Hr motility scores

Ejaculates	Bull				Ram			
	Buffered Glucose Sodium Chloride		0·5 Per Cent. Crude Lecithin		Buffered Glucose Sodium Chloride		0·5 Per Cent. Crude Lecithin	
	Control	Shocked	Control	Shocked	Control	Shocked	Control	Shocked
1	2·5	0·5	2·5	2·3	3·5	2·5	3·5	3·5
2	3·5	2·0	3·5	3·0	2·8	2·0	2·8	2·8
3	3·0	1·5	3·0	3·0	4·0	2·3	4·0	3·0
4	3·0	2·0	3·0	3·0	2·5	1·5	2·5	2·0
5	2·8	0·0	2·8	2·5	3·5	2·0	3·5	2·8
6	2·5	1·8	2·5	1·8	2·8	1·5	2·8	2·5
7	2·5	0·5	2·8	3·3	3·5	1·5	3·3	2·8
8	2·8	1·5	3·0	3·0	2·8	2·0	2·8	2·8
9					2·5	1·5	2·8	2·8
10					3·0	1·5	3·0	2·8
Totals	22·6	9·8	23·1	21·9	30·9	18·3	31·0	27·8
Mean score	2·8	1·2	2·9	2·7	3·1	1·8	3·1	2·8

In order to find out the nature of any active substance in egg yolk, several extracts were made. Egg yolks were extracted several times in a blender with acetone, alcohol, and ether in succession. Exhaustive extraction was not attempted and the use of heat was avoided, all extractions being made at room temperature. The various extracts and residues were retained and dried *in vacuo* to remove all traces of the solvents.

The solvent-free products were suspended in the glucose-sodium chloride diluent and tested for capacity to prevent cold shock. The quantity of material obtained from the ether extraction was extremely small and no tests were made with it. The results are given in Table 2 (bull and ram). Analyses of variance of the results showed significant diluent and ejaculate effects with both species. Comparisons were made between the glucose-sodium controls (1) and the corresponding cold-shocked group (2) and gave $t_{(21)} = 6\cdot5$, $P < 0\cdot001$ (bull) and $t_{(35)} = 8\cdot6$, $P < 0\cdot001$ (ram). Further comparisons were made between the control group (1) and the acetone-soluble group (5), $t_{(21)} = 6\cdot9$, $P < 0\cdot001$ (bull) and $t_{(35)} = 6\cdot8$, $P < 0\cdot001$ (ram) and also between (1) and the insoluble residue (8), $t_{(21)} = 4\cdot6$, $P < 0\cdot001$ (bull) and $t_{(35)} = 7\cdot7$, $P < 0\cdot001$ (ram). Other comparisons were made but the differences were not significant. These results indicated that the activity of egg yolk in preventing cold shock, was to be found in the alcohol-soluble fractions.

TABLE 4

EFFECT OF 0·5 PER CENT. PURE LECITHIN ON THE DEGREE OF COLD SHOCK

½ Hr motility scores

Ejaculates	Bull				Ram			
	Buffered Glucose Sodium Chloride		0·5 Per Cent. Pure Lecithin		Buffered Glucose Sodium Chloride		0·5 Per Cent Pure Lecithin	
	Control	Shocked	Control	Shocked	Control	Shocked	Control	Shocked
1	3·0	2·0	3·0	3·0	2·8	2·0	2·5	2·3
2	2·8	0·0	2·8	2·3	2·8	0·0	2·8	2·3
3	3·0	1·5	3·0	2·8	3·3	2·0	2·8	2·8
4	2·8	1·8	2·5	2·3	2·8	2·3	3·0	2·8
5	2·8	2·3	2·8	3·0	4·0	0·5	4·0	1·0
6	3·0	2·3	3·0	3·0	4·0	1·0	4·0	2·3
7	3·3	2·3	3·3	2·8	3·5	0·5	3·0	1·0
8	2·5	1·5	2·5	2·5	4·0	1·0	4·0	2·8
9	2·3	1·0	2·8	2·5	2·8	1·3	2·8	2·0
10	2·8	0·0	2·5	2·5	3·5	2·0	4·0	2·8
11	2·5	1·3	3·3	3·0	3·3	1·3	3·3	2·8
12	2·5	2·0	3·0	3·0	3·5	1·0	3·0	2·5
13	2·5	1·8	3·0	3·0	3·5	1·0	3·5	2·3
14					3·5	2·0	3·8	2·8
Total	35·8	19·8	37·5	35·7	47·3	17·9	46·5	32·5
Mean score	2·7	1·5	2·9	2·7	3·4	1·3	3·3	2·3

Further tests were made using the acetone precipitate of the alcohol-soluble extract (Table 3), and *t*-tests were made comparing the two cold-shocked groups which gave $t_{(7)} = 4\cdot8$, $P < 0\cdot01$ (bull), and $t_{(9)} = 10\cdot5$, $P < 0\cdot001$ (ram), clearly indicating the superiority of the yolk extract.

The fatty nature of the active extract, insolubility in acetone, and solubility in alcohol suggested that an active substance in egg yolk might be lecithin, which is the principle phospholipid present (Romanoff and Romanoff 1949). Pure lecithin was prepared from egg yolks by the method of Levene and Rolf (1927) and later by that of Pangborn (1951), the latter being preferred because of the relative ease of preparation. The lecithin was stored under acetone in the refrigerator and before use was dried *in vacuo* for at least ½ hr before suspension in buffered glucose-sodium chloride.

Table 5

LECITHIN CONCENTRATION AND THE DEGREE OF COLD SHOCK

½ Hr motility scores

Bull Ejaculates	Control	Cold Shocked				
		Glucose Sodium Chloride	Lecithin (% w/v)			
			0·50	0·25	0·12	0·06
1	2·3	1·3	2·5	2·5	2·8	1·3
2	3·5	2·8	3·5	3·8	3·5	3·8
3	2·8	2·0	2·5	2·5	2·5	2·3
4	2·5	2·0	2·5	2·5	2·5	2·5
5	2·8	1·0	3·3	3·3	2·8	2·8
6	2·8	1·5	3·0	3·0	2·8	2·3
Totals	16·7	10·6	17·3	17·6	16·9	16·2
Ram Ejaculates						
1	4·0	1·0	1·0	2·0	2·0	2·5
2	3·5	1·0	1·5	2·5	2·5	2·0
3	4·0	2·3	3·3	3·3	3·5	3·0
4	4·0	2·8	3·5	3·5	3·3	3·5
5	3·5	1·0	2·8	3·3	3·3	3·3
6	3·5	1·0	2·8	3·0	2·8	2·5
Totals	22·5	9·1	14·9	17·6	17·4	16·8

Tests of the pure lecithin were made (Table 4), the results of a *t*-test between the two cold-shocked groups indicating ability of lecithin to prevent cold shock, $t_{(12)} = 5{\cdot}5$, $P < 0{\cdot}001$ (bull), and $t_{(13)} = 6{\cdot}5$, $P < 0{\cdot}001$ (ram). It may be noted, however, that protection with ram spermatozoa was far from complete and comparison of the two lecithin-treated groups gave $t_{(13)} = 2{\cdot}8$, $P < 0{\cdot}02$.

The effects of various levels of lecithin were observed (Table 5) and analysis showed no significant differences between levels in both species. All levels of lecithin afforded complete protection from cold shock with bull spermatozoa but in the case of the ram, as shown by comparison of the 0·25 per cent.

level with the glucose-sodium chloride control ($t_{(25)} = 3{\cdot}3$, $P < 0{\cdot}01$) only partial protection occurred.

All the previous experiments were carried out at a pH of 7·0 to 7·2, but subsequently it was found that pH played an important part in the action of lecithin in preventing cold shock with ram spermatozoa, although this does not appear to be so with bull spermatozoa.

TABLE 6

EFFECT OF pH ON THE ACTION OF 0·25 PER CENT. LECITHIN IN PREVENTING COLD SHOCK IN RAMS

½ Hr. motility scores

	0·25 Per Cent. Lecithin			Buffered Glucose Sodium Chloride
Nominal pH	6·5	7·2	8·0	7·2
Range	6·2-6·6	7·0-7·2	7·4-7·7	6·9-7·2
Ejaculates				
1	3·5	2·5	2·3	0·5
2	3·3	2·3	2·0	1·0
3	3·0	1·5	1·0	1·3
4	2·5	2·0	1·0	0·5
5	3·3	2·3	2·0	0·5
6	3·3	2·0	2·0	1·5
7	2·8	1·5	2·0	0·0
8	2·3	2·3	2·0	0·0
9	2·8	1·3	1·0	0·0
10	4·0	3·0	2·5	2·0
11	3·0	2·0	1·0	1·5
12	3·5	2·5	1·5	1·5
13	3·3	2·5	1·5	1·5
14	3·3	2·8	1·0	1·0
15	2·8	2·0	2·0	1·5
Totals	46·7	32·5	24·8	14·3
Mean score	3·1	2·2	1·7	1·0

Lecithin at 0·25 per cent. concentration was suspended in a diluent containing 3 per cent. glucose, 0.26 per cent. sodium chloride, and buffered with 0·1M sodium phosphates, which were added at the rate of 20 ml buffer per 100 ml total diluent. The nominal pH levels used were 6·5, 7·2, and 8·0, variations are recorded in Table 6, which contains the results from 15 ram ejaculates. Analysis of variance of the results is given in Table 7 and clearly shows that pH 6·5 gave best results, protection from cold shock being greatly decreased above pH 7·2.

IV. Discussion

The occurrence of cold shock has been demonstrated in the spermatozoa of several species, and means to avoid it by slow cooling or the addition of egg yolk have been suggested. The observations presented here point to the phospholipid lecithin as a factor in egg yolk which will considerably reduce the damage caused by cold shock, as judged by motility observations. The good protection afforded to ram spermatozoa at the rather low pH 6·5 is somewhat less than that for optimal spermatozoal activity (pH 6·9) but this may be related to the isoelectric point of lecithin which Bull and Frampton (1936) give as pH 6·4.

The nature of the change in the cell caused by sudden temperature changes is unknown, but Walton (1947) believes it to be a surface phenomenon, involving damage to the motor mechanism and flagellar surface. The loss of resistance to cold shock which occurs on ejaculation may support this view, especially as Milovanov (1934) states that the accessory secretions remove the lipoid capsule from the spermatozoa. The presence of egg yolk or lecithin may repair the deficiency.

Table 7

SUMMARY ANALYSIS OF VARIANCE OF THE DATA IN TABLE 6

Source of Variation	Degrees of Freedom	Variance Ratio†
Ejaculates	14	3·6*
Diluents		
1. Lecithin *v.* no lecithin	1	122·0*
2. pH 6·5 *v.* 7·2, and 8·0	1	85·2*
3. pH 7·2 *v.* 8·0	1	11·6*
Residual	42	*0·17*

*$P < 0{\cdot}01$.

†The actual value of the residual variance is given in the last column in italics.

The occurrence of phospholipids in human spermatozoa has been demonstrated by Wislocki (1950) and Brown (1952), the midpiece in particular showing their presence. More recently Boguth (1952) has investigated the plasmalogen content of egg yolk and bull semen and has found that the 20 per cent. egg yolk used in semen diluents may be completely replaced by 0·1 per cent. of phosphatides derived from the egg yolk. The total content of plasmalogen in bull semen is given as 0·3 to 0·9 mg/ml, of which one-third is found in the seminal plasma.

The observations recorded in this report are in accord with the brief account of Walton (1947) but differ in all respects from those of Mayer and Lasley (1944, 1945). The active substance isolated by them was a non-lipoidal white crystal-like material, while the acetone-insoluble, alcohol-soluble fraction of egg yolk, described as toxic, contains principally lecithin and cephalin which have been amply shown as very beneficial to spermatozoa. No explanation of this apparent contradiction can be given. However, recently Kampschmidt, Mayer, and Herman (1953) state that lipoprotein and the phospholipids which occur in egg yolk will protect bull spermatozoa from cold shock, but the evidence presented for the action of the phospholipids is not clear. Also the lipoprotein will replace egg yolk for the storage of spermatozoa.

The part played by phospholipids in the physiology of the cell is not clear, but it has been shown (Lardy and Phillips 1941*a*) that egg lecithin decreases the respiration and greatly prolongs the motility of bull spermatozoa in Ringer phosphate; oxygen consumption is not appreciably increased by lecithin added to a glucose medium, and this Lardy and Phillips (1941*b*) state is an indication that the intracellular reserves of the spermatozoa are phospholipid in nature. Sea urchin spermatozoa also utilize endogenous phospholipid as a source of energy (Rothschild and Cleland 1952).

Milovanov and Selivanova (1932) found that lecithin improves the viability of stored bull semen while Phillips and Spitzer (1946) recommend the use of lecithin in their synthetic pabulum for bull semen; the fertility rates for the latter being equal to those obtained using the normal egg yolk diluent. It would thus seem reasonable to use lecithin for the prevention of cold shock, with the expectation of no undesirable side effects.

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